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パラミキソウイルスNPタンパク質のRNAポリメラーゼの機能解析

Analysis of RNA polymerase activity associated with NP protein of paramyxovirus

大阪大学微生物病研究所神経ウイルス分野
Department of Neurovirology, Research Institute for Microbial Diseases, Osaka Univ.

眞砂 明典
Akinori Masago

(指導：上田重暗教授)

(平成12年1月31日受付)

During the life cycle of Sendai virus (SeV), three types of viral RNA (mRNA, full-length genome and antigenome RNA) are synthesized in the cytoplasm by the viral nucleocapsid proteins (NP, P and L). Although L protein or the complex of P and L proteins have been assigned to viral RNA polymerase which utilize the NP protein-RNA complex as a template, detail mechanism of the viral RNA synthesis is still obscure. In this study, we established a cell line expressing T7 RNA polymerase stably (LLCMK$_2$#T7) and investigated a function of NP protein of SeV.

We constructed a SeV mini-genome (pHVLuciB), which directed the synthesis of (-) strand RNA consisting of SeV genome 3' leader-luciferase-SeV 5' trailer under T7 RNA polymerase promoter. When pHVLuciB was transfected to LLCMK$_2$#T7 cells together with pGEMNP (NP protein expression vector), luciferase activity and (+) strand luciferase RNA were detected in the transfected cells. (+)Strand RNA synthesis required the expression of full-sized NP protein and the (-) strand template with genome 3' leader. These results suggest that SeV NP protein has intrinsic RNA polymerase activity that directs the synthesis of (+)strand RNA.

Key Word: Sendai virus, NP protein, RNA polymerase, Nucleocapsid

目　的

パラミキソウイルス科のウイルス（以下、パラミキソウイルス）は麻疹ウイルス、流行性耳下腺炎ウイルス、パラインフルエンザウイルスなど人間に病原性を持つものが多い。また、パラミキソウイルスのプロトタイプと考えられているセンダイウイルス（SeV）は近年遺伝子治療用ベクターとしても注目されている。

パラミキソウイルスは１本の(-)鎖のRNAを持つウイルスであり、約15Kヌクレオチドのゲノムを持っている。DNAウイルスや(+)鎖RNAウイルスとは異なり、パラミキソウイルスの(-)鎖ゲノムRNAは細胞に導入してもそのままではウイルスはできない。cDNAからウイルスを作成するためには、T7 RNAポリメラーゼを発現する組み換えワクシニアウイルス（RVVT7）をLLCMK$_2$細胞に感染させた後、T7プロモーターをもつ鋳型cDNAを導入し、同時にNP、P、L遺伝子をトランスに供

給することが必要である[1]。その理由としては、ゲノム(−)鎖RNAがNPタンパク質と結合した複合体を鋳型として、P及びLタンパク質の複合体から成るRNAポリメラーゼがmRNAの転写と(+)鎖アンチゲノムRNAの合成を行うためだと考えられている[2),3)]。本研究では、安定にT7 RNAポリメラーゼを発現する細胞（LLCMK$_2$#T7）を樹立し、パラミキソウイルスのヌクレオキャプシドタンパク質の機能解析を試みた。

材料と方法

細胞培養

本研究で用いたLLCMK$_2$、LLCMK$_2$#T7細胞は10％新生仔牛血清（NCS）、イーグル最小必須培地（MEM, Gibco BRL）中で37℃、5％CO_2の条件下で培養した。

LLCMK$_2$#T7細胞：9.25μgのプラスミドpSV2-neoと0.75μgのプラスミドpCAT7（T7 RNA ポリメラーゼ発現ベクター）に対してLipofectin（Gibco BRL）100μlの割合で混合し、1.5mlのHBSで希釈してLLCMK$_2$細胞に37℃、4時間インキュベートした後、培地10mlの10％NCSを含むMEMに交換して2日間培養した。これらの細胞を2週間1.2mg/mlの G418存在下で培養した後、出現した個々の G418耐性細胞クローンを単離し、細胞抽出液のT7 RNAポリメラーゼ活性を測定して，タンパク質４μｇ当たり１０UのT7 RNAポリメラーゼ活性を持つLLCMK$_2$#T7細胞を得た。

プラスミドの作成

以下の組換えプラスミドDNAは標準的方法[4)]とオリゴヌクレオチドを使ったin vitro mutagenesis[5)]により構築した。

ミニゲノム鋳型（pHVLuciB）（Fig1）：T7プロモーター下流にSeVゲノム3'及び5'末端の相補的な配列の間にルシフェラーゼcDNAを挿入し、さらにSeVゲノム3'末端に制限酵素BsaI部位を挿入して、T7 RNAポリメラーゼ存在下で(−)鎖ルシフェラーゼRNAを合成するプラスミドを作成した。

pGEMNP改変プラスミド：NPタンパク質をコードするmRNAをT7 RNAポリメラーゼにより合成する鋳型pGEMNPは、Dr. Kolakofskyにより分与された。

pGEMNPのT7プロモーターとNP遺伝子の間に開始コドン、終止コドンを挿入したプラスミド、T7プロモーターを欠損させたプラスミドを作成した。

SeV5'、SeV3'欠損ミニゲノム鋳型：それぞれSeV5'末端より138bp、SeV3'末端より114bpを欠損させたプラスミド、あるいはSeV5'、3'末端両方を欠損させたプラスミド、さらにSeV5'、3'末端とは関係のない塩基配列で置換したプラスミドを作成した。

トランスフェクション

LLCMK$_2$細胞を60mmディッシュに1.0x10^{6}細胞接種し、18時間後、pHVLuciBとヌクレオキャプシドタンパク質発現ベクター（pGEMNP、pGEMP、pGEML）及びpAct-LacZ（β−ガラクトシターゼ発現ベクター）（全部でDNA6μg）とDOTAP（Boehringer-Mannheim）45μlを加えた4mlのOpti-MEMで37℃、4時間インキュベートした後、4mlの10％NCSを含むMEMに交換して22時間培養した。

その他

ルシフェラーゼアッセイ[6)]、β−ガラクトシターゼアッセイ[7)]は既報に従って行った。

結　果

安定にT7 RNAポリメラーゼを発現しているLLCMK$_2$#T7細胞に、ミニゲノム鋳型（pHVLuciB）とヌクレオキャプシドタンパク質発現ベクター（pGEMNP、pGEMP、pGEML）を様々な組合せで同時にトランスフェクションし、ルシフェラーゼの発現比較を行った。この際、トランスフェクションの効率を測るために、β-ガラクトシターゼ発現ベクターを同時に導入し、ルシフェラーゼ活性をβ-ガラクトシターゼ活性で割ってルシフェラーゼ比活性を出した。この結果、pGEMNPを導入した場合に常に高いルシフェラーゼ活性が検出された（TableI）。

次ぎにNPタンパク質の発現が(+)鎖RNA合成に必須か否か確認するために、pGEMNPを改変したプラスミドを作製した。pGEMNP、pGEMNPのT7プロモーターとNP遺伝子の間に開始コドン、終

止コドンを挿入したプラスミド、pGEMNPのT7プロモーターを欠損させたプラスミドをそれぞれ鋳型pHVLuciBと一緒に細胞にトランスフェクションし、ルシフェラーゼの発現比較を行い、(+)鎖RNAの合成とNPの発現の相関を検討した。その結果、anti-NP polyclonal antibodyを用いたProtein blottingで検出される分子量60KDaのNPタンパク質が検出される時にのみ、(+)鎖RNAが合成されることを確認した（Fig.2）。

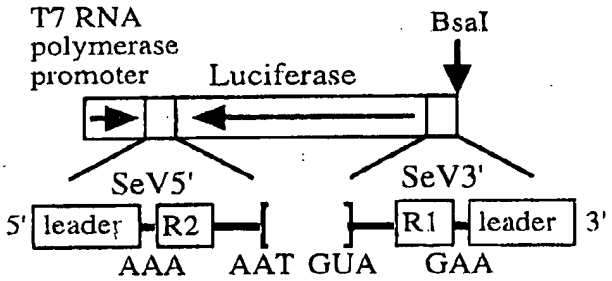

Fig1. SeV mini genome(pHVLuciB)

	NP	P	L	pHVLuciB	Luci/β gal
1	+	+	+	+	723.0
2	+	+	-	+	268.9
3	+	-	+	+	423.8
4	-	+	+	+	43.9
5	+	-	-	+	279.0
6	-	+	-	+	5.2
7	-	-	+	+	5.0
8	-	-	-	+	0.1
9	+	-	-	-	0.0

Table 1. Detection of luciferase activity in the cells transfected with SeV mini genome and NP, P and L expression plasmids.
pHVLuciB=1 μg, pGEMNP=2 μg, pGEMP=1 μg and pGEML=1 μg

また、NPタンパク質のもつRNAポリメラーゼ活性に鋳型特異性があるかどうか検討するために、ミニゲノム5'と3'末端をSeVとは関係のない塩基配列で置き換えた鋳型を用いて、NPによる(+)鎖RNAの合成を検討したところ、ルシフェラーゼ活性が著しく低下した。さらに5'あるいは3'末端、5'と3'末端の両方を削ったミニゲノムを用いて、NPタンパク質による(+)鎖RNAの合成を検討した結果、3'末端を削った場合にルシフェラーゼ活性が大きく低下していることから、(−)鎖RNAのうち、SeVゲノム3'末端に相当する配列が(+)鎖RNAの合成に必要であることがわかった（Fig.3）。

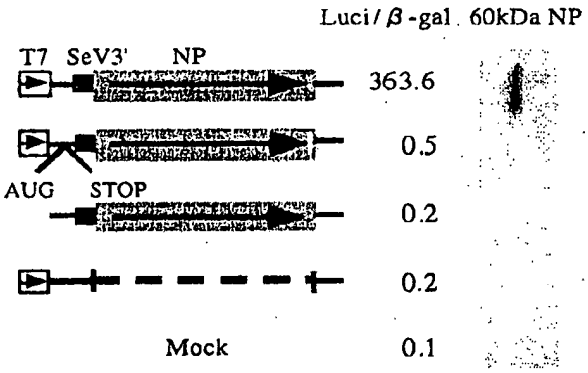

Fig 2. Ability of pGEMNP derivatives to synthesize (+) strand luciferase RNA.

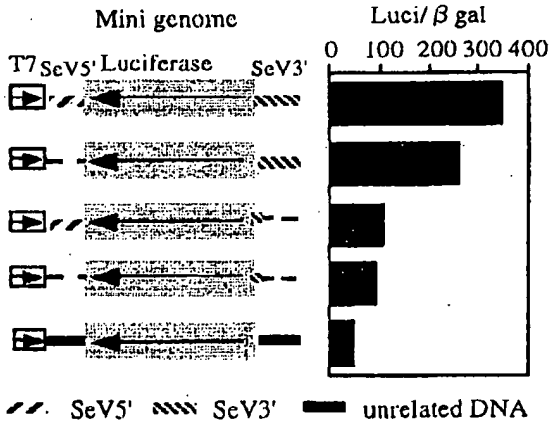

Fig 3. (+) strand RNA synthesis from pHVLuciB derivatives.

考　察

今までSeVの転写・複製にはNP、P、L遺伝子が

必要であり、NPタンパク質はRNAと結合し、これを細胞内のRNaseから保護している機能を持ち、Pタンパク質とLタンパク質の複合体がNP-RNA複合体を基質としてRNA依存性RNAポリメラーゼとして働くと考えられてきた。しかし、今回の実験で、NPタンパク質とミニゲノムを同時に発現させた細胞ではP、Lタンパク質がなくてもミニゲノム由来の(-)鎖RNAから(+)鎖RNAが合成されることが確認された。またRNA合成の開始点がR1領域にあることを確認した（data not shown）。これらの結果は、ヌクレオキャプシドの主要構成成分であるNPタンパク質自体にセンダイウイルスの(-)鎖ゲノムRNAを鋳型として(+)鎖RNAを合成するRNA依存性RNAポリメラーゼ活性があり、この活性がウイルスmRNAの転写に関与していることを強く示唆している。これまで他の研究グループからNPタンパク質のN末端400アミノ酸がSeVの転写・複製に必要であることが報告されているため[8),9)]、RNAポリメラーゼの活性部位はこの中にある可能性が高い。今後、NPタンパク質の持つRNAポリメラーゼ活性をさらに詳細に調べるために、NPタンパク質を含む細胞抽出液を用いて*in vitro*でミニゲノム由来の(-)鎖RNAから(+)鎖RNAの合成を行う系を確立すると共に、さらにNPタンパク質の(+)鎖RNAの合成能がこのタンパク質のどの領域にあるのかをdeletion mutantを用いて決定していきたい。

謝　辞

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抄　録

パラミキソウイルスの転写・複製にはNP、P、Lタンパク質が必要であり、ゲノム(-)鎖RNAがNPタンパク質と結合した複合体を鋳型として、P及びLタンパク質の複合体から成るRNAポリメラーゼがmRNAの転写と(+)鎖アンチゲノムRNAの合成を行うと考えられてきた。しかし実際にどのタンパク質にRNAポリメラーゼ活性が存在し、それがどのように調節されてウイルスの転写、複製が制御されているのかは明らかではない。

本研究ではT7 RNAポリメラーゼを安定に発現する細胞を樹立して、NPタンパク質にミニゲノムの(-)鎖RNAから(+)鎖RNAを合成するRNAポリメラーゼ活性が存在することを見い出した。

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) version of SeV in which the accessory V gene had been knocked ut. Based on these results, we discuss the utility of SeV vector in terms of both efficiency and safety.

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New approaches to the development of virus vaccines for veterinary use

K. Yamanouchi [1], T. Barrett [2] & C. Kai [3]

(1) Nippon Institute for Biological Science, 2221–1 Shin-machi, Ome, Tokyo 198, Japan
(2) Institute for Animal Health, Pirbright Laboratory, Ash Road, Woking, GU24 0NF, United Kingdom
(3) Department of Veterinary Microbiology, Faculty of Agriculture, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan

Summary

The marked progress in recombinant deoxyribonucleic acid (DNA) technology during the past decade has led to the development of a variety of safe new vaccine vectors which are capable of efficiently expressing foreign immunogens. These have been based on a variety of virus types – poxviruses, herpesviruses and adenoviruses – and have led to the production of many new potential recombinant vaccines. Of these recombinant vaccines, the rabies vaccine, in which the rabies G protein is expressed in a vaccinia vector, has been widely used in the field to prevent the spread of rabies both in Europe and in the United States of America. A recombinant Newcastle disease virus vaccine, using fowlpox virus as the vector to express immunogenic proteins from the Newcastle disease virus, has been licensed as the first commercial recombinant vectored vaccine. Many other recombinant virus vaccines are still at the stage of laboratory or field testing.

The most recent breakthrough in vaccinology has been the success with the use of naked DNA as a means of vaccination. This approach has shown great promise in mouse model systems and has now become the most active field in new vaccine development. Molecular redesigning of conventional ribonucleic acid (RNA) viruses to obtain more stable attenuated vaccines was previously possible only for positive-strand RNA viruses, such as poliovirus. However, recent advances in molecular biological techniques have enabled the rescuing of negative-strand viruses from DNA copies of their genomes. This has made it possible to engineer specific changes in the genomes of Rhabdoviridae and Paramyxoviridae, both of which include several viruses of veterinary importance. The authors describe the current progress in the development of vector vaccines, DNA vaccines and vaccines based on engineered positive- and negative-strand RNA virus genomes, with special emphasis on their application to diseases of veterinary importance.

Keywords

Adenovirus vector – Deoxyribonucleic acid vaccine – Herpesvirus vector – Poxvirus vector – Recombinant vaccine – Rescue system – Vector vaccine.

Introduction

In 1796, Edward Jenner conducted the first smallpox vaccination using material obtained from a lesion on a cow suffering from cowpox. Subsequently, the vaccine was maintained by arm-to-arm passage until the mid-1840s, when the technique for the production of smallpox vaccine on the skin of calves or sheep was developed by Negri, a medical doctor in Naples (5). This was the first use of animals for large-scale vaccine production. Thereafter, numerous vaccines for both human and animal use were produced in animals.

The next breakthrough in technology came in the mid-1950s, when chicken embryos and tissue culture cells were adapted for the production of vaccines. However, the basic concept

remained unchanged, i.e. virus growth in animals or cells derived from animals. It is noteworthy that the global eradication of smallpox, a landmark in the history of microbiology, was achieved using only the oldest type of vaccine, produced in the skin of calves and sheep.

Advances achieved in recombinant deoxyribonucleic acid (DNA) technology in the 1970s opened a new era in vaccine development. Instead of growing viruses in animals or cells, individual protective antigens could be expressed in large quantities in mammalian or insect cells or in bacteria, the so-called second generation of subunit vaccines. Owing to an inadequate immune response to viral proteins produced in this way, very few successes have been achieved, in spite of great efforts over the past 20 years. However, recombinant DNA technology also made it possible to use safe virus vectors for the expression of protective antigens from dangerous pathogens, i.e. recombinant vector vaccines. Unlike the recombinant subunit vaccines, recombinant vector vaccines have been shown to result in highly immunogenic responses to the foreign protein. A recombinant rabies vaccine, which uses vaccinia virus as the vector, has already been widely used in areas of endemic rabies in Western Europe. Many other recombinant vector vaccines have proved their usefulness in laboratory trials, and great efforts are being made to improve their safety for field use.

Most recombinant vaccines have been based on DNA virus (poxvirus) vectors but another approach has been to use conventional ribonucleic acid (RNA) virus vaccines, such as the poliovirus vaccine, to express immunogenic epitopes from other viruses for which no effective live attenuated vaccines exist. This is a relatively easy task, since the virion RNA produced from the modified DNA copy of the genome of positive-strand virus is infectious when injected into susceptible cells. In the case of negative-strand viruses, the task is more difficult since the genome RNA is not infectious. However, recent advances in molecular techniques have enabled this difficulty to be overcome, and it is now possible to produce infectious virus from DNA copies of RNA viruses, such as rabies, Sendai virus, measles, rinderpest and respiratory syncytial virus (13). Extra reading frames have been incorporated into these genomes and it is now possible to produce genetically defined and marked vaccines and to use these viruses as vectors for other immunogenic proteins.

In 1990, a unique approach to vaccination was reported, i.e. the use of naked DNA encoding immunogenic proteins as vaccines. Over the past seven years, DNA vaccines have been produced for a variety of diseases and tested in the laboratory with considerable success. These advances in molecular technology have created new opportunities to produce safer, genetically defined vaccines for both human and veterinary use. In this paper the present situation regarding the development and testing of virus vector vaccines and DNA vaccines, as well as the potential usefulness of the rescue system of negative-strand RNA viruses, will be reviewed.

Vector vaccines based on deoxyribonucleic acid viruses

Following the demonstration in 1982 that foreign antigens could be expressed using vaccinia virus (26, 35), many attempts have been made to develop a variety of new vaccines using vaccinia virus as a vector, especially in the field of veterinary medicine. Table I summarises the examples of vector vaccines developed for veterinary use.

At first, the smallpox vaccine, which was used in the campaign for the global eradication of smallpox, was used as the vector. However, the low morbidity and mortality rate associated with smallpox vaccination was considered to pose too great a risk to make such recombinant vaccines acceptable for widespread human use. This is particularly the case in areas of the world where large sections of the population are immunocompromised due to high rates of human immunodeficiency virus (HIV) infection. More attenuated strains of the vaccine were investigated and, more recently, vaccinia virus attenuated by genetic manipulation was constructed, in the search for suitable vectors for the expression of immunogenic foreign proteins for veterinary and human use. The concept of vector attenuation has been developed still further by the use of poxviruses that do not replicate in the target host, i.e. avipox viruses in mammals. Other large DNA viruses, such as the herpesviruses, have been used as vectors for particular animal species. These include Aujeszky's disease (pseudorabies) virus for use in pigs, herpesvirus of turkeys and feline and canine herpesviruses.

Adenovirus, a smaller DNA virus, has also been used as an expression vector. However, due to technical difficulties, particularly the tight constraints on the size of DNA insert that can be accommodated in the genome, construction of recombinant adenoviruses has been limited. Adenoviruses expressing foreign proteins are considered suitable as vectors in 'gene therapy' and this has led to technical improvements in the vectors now available. In the future, adenovirus-recombinant vaccines may be more widely tested, particularly as they have the potential to elicit a strong mucosal immune response.

Recombinant vaccines based on mammalian poxvirus vectors

Although the pioneering work on recombinant vaccine production was conducted using vaccinia virus, the only vaccinia-based recombinant virus licensed as a veterinary vaccine is the rabies-recombinant vaccine. This vaccine is used for the oral vaccination against rabies of foxes in Europe and raccoons and striped skunks in North America; these species being the most important natural reservoirs of rabies

Table I
Vector vaccines for veterinary use

Vaccine	Vector	Inserted gene	Remarks	Reference
Rabies	Vaccinia (Copenhagen)	G	Widely used in the field	(9)
Rinderpest	Vaccinia (LC16mO)	H	Ready for field testing	(57)
	Vaccinia (Wyeth)	H, F		(19)
	Capripox	H, F	Also effective on lumpy skin disease	(32)
Bovine leukaemia	Vaccinia (LC16mO)	env		(34)
Aujeszky's disease (pseudorabies)	Vaccinia (NYVAC)	gD		(10)
Aujeszky's disease	Swinepox	gp50, gp63	Partial protection	(50)
Avian influenza	Fowlpox	HA		(47)
Newcastle disease	Fowlpox	HN	Also effective on fowlpox. Licensed	(27)
		F	Also effective on fowlpox	(48)
Infectious bursal disease (Gumboro disease)	Fowlpox	VP2	Also effective on fowlpox	(4)
Rabies	Canarypox	G	Non-replicative	(17)
Viral haemorrhagic disease of rabbits	Canarypox	Capsid	Non-replicative	(15)
Classical swine fever	Aujeszky's disease	gpE1	Also effective on Aujeszky's disease	(51)
Feline calicivirus	Feline herpes 1	Capsid		(60)
Feline leukaemia	Feline herpes 1	gp70/p15		(52)
Newcastle disease	Herpesvirus of turkeys	HN	Also effective on Marek's disease	(31)
Viral haemorrhagic disease of rabbits	(Marek's disease vaccine)			
	Myxoma	Capsid	Also effective on myxomatosis	(6)
Rabies	Adeno 5	G	Oral immunisation	(58)

on their respective continents. The recombinant rabies vaccine was developed by inserting the glycoprotein G gene of rabies virus into the Copenhagen strain of vaccinia virus, one of the strains used as a smallpox vaccine (7). The greater resistance to environmental temperature fluctuations of vaccinia recombinants, in relation to the naturally attenuated rabies strain used in domestic dogs, makes them ideal vaccines for spiking baits, which are then distributed from the air. The vaccine maintains its potency for many months, even in conditions where it is frequently frozen and thawed. Previous attempts to control sylvatic rabies in Europe using attenuated rabies in baits for oral vaccination were not very effective, due to vaccine instability. The recombinant vaccine very effectively solved this problem.

The demonstration of successful oral vaccination of foxes with the rabies-recombinant vaccine in 1986 was followed by extensive investigations into the safety of this vaccine for domestic, laboratory and wild animals. Tests were first conducted under laboratory conditions. These were followed by carefully controlled and monitored field tests performed in Western Europe to vaccinate wild foxes. Similar tests, in which raccoons and coyotes were vaccinated, were later conducted in the United States of America (USA). Between 1989 and 1995, approximately 8.5 million doses were dispersed in these areas without any problems, demonstrating the effectiveness of wildlife vaccination using recombinant vaccines on a large scale. These field studies have been described in detail in a recent review (9). An important feature of the campaign was to persuade the public that the vaccine was safe and so gain public approval for the widespread use of vaccinia-based recombinant vaccines (36).

Other important veterinary diseases, for which the existing vaccine is unstable or safely attenuated strains of the pathogen are not available, could also benefit from the use of recombinant vaccines. Rinderpest is one of the most important contagious viral diseases of domestic animals, affecting mainly cattle and buffalo. A major obstacle to the campaign to eradicate rinderpest is the heat-labile nature of the current rinderpest vaccine, despite its very high efficacy. With the aim of producing a more heat-stable vaccine, three different recombinant rinderpest vaccines (RRVs) have been produced.

The first expresses the H protein gene of rinderpest virus, using the LC16mO attenuated smallpox vaccine strain as the vector (57). The second expresses both the H and F protein genes of rinderpest virus, using the Wyeth smallpox vaccine strain as the vector (19). The third is a mixture of two separate recombinants, which expresses either the H or the F protein gene of rinderpest virus, using an attenuated vaccine strain of capripox virus as a vector (32). The RRV (LC16mO) vaccine has been extensively tested under laboratory conditions to establish its efficacy in protecting against challenge with virulent rinderpest virus. Its safety in cattle, genetic stability through several passages in cattle and long-lasting immunity,

greater than one year, were confirmed (22). This vaccine (LC16mO) was also shown to be highly heat-stable when lyophilised – a stability comparable to that of the original smallpox vaccine – and is now ready for testing under field conditions. The RRV (Wyeth) has similarly been shown to protect cattle from challenge with virulent rinderpest virus in preliminary short-term trials (19). The RRV (capripox) has the added advantages that it can be used to protect cattle against both rinderpest and lumpy skin disease and is non-pathogenic for humans, making it extremely safe to handle. One disadvantage associated with the RRV (capripox) vaccine is the interference with the efficacy of the vaccine caused by pre-existing anti-capripox antibodies, a factor which was not a problem in the case of RRV (LC16mO) (32, 56).

Swinepox, another mammalian pox virus, has been considered as a vector for use in pigs. The disease caused by swinepox virus in pigs is relatively benign, its natural host range is restricted to this species and it is found worldwide. Based on these ideal vector characteristics, a recombinant Aujeszky's disease vaccine was developed. The glycoproteins (gp) gp50 and gp63 of Aujeszky's disease virus were inserted into the thymidine kinase gene of swinepox virus, under the control of the vaccinia p7.5K promoter. However, only partial protection of pigs against challenge with virulent Aujeszky's disease virus was conferred by this recombinant vaccine (50).

Further attenuation of the vaccinia virus vector

Generalised vaccinia and encephalitis were complications of vaccination in one out of 300,000 people vaccinated during the global smallpox eradication campaign conducted in the 1970s, and the risks may be greater in immune-compromised people. Since the end of mass smallpox vaccination, the number of people susceptible to infection with vaccinia virus is increasing and the possibility exists of cross-infection from animals vaccinated with vaccinia-recombinant vaccines. During the smallpox eradication campaign, four highly attenuated strains of smallpox virus were developed for use as vaccines. These were the LC16mO and LC16m8 strains (21), the modified Ankara (MVA) strain (29) and the CV-1 strain (24).

Both LC16mO and LC16m8 were derived from the Lister strain by selecting a temperature-sensitive mutant, which was then grown in rabbit kidney (RK) 13 cells. These strains were shown to be attenuated in human trials but only the latter was licensed as an attenuated smallpox vaccine.

The attenuation of the MVA strain was also confirmed in large-scale human trials. Since this virus was passaged over 570 times in chicken embryo fibroblasts, it does not productively infect mammalian cells and must be grown in avian cells (42), making it an ideal vaccinia strain for use as a recombinant vaccine vector.

The CV-1 strain was passaged in chicken embryo organ culture and on the chorioallantoic membranes of chicken embryos more than 70 times in total. In a comparative study of subcutaneous inoculation in humans, however, this virus was found to be less likely to induce antibody, compared with the Lister and other strains (12). As a result of the high immunogenicity of the LC16mO strain in rabbits and cattle, the recombinant rinderpest vaccine was developed using this vaccinia strain as the vector, and its efficacy and safety were confirmed, as described in the previous section (see 'Recombinant vaccines based on mammalian poxvirus vectors', above). The MVA strain was used to make a recombinant influenza vaccine expressing the haemagglutinin and nucleoprotein genes of influenza virus, and protective immunity was demonstrated in mice (45).

Attenuation of a conventional smallpox vaccine (Copenhagen strain) has been attempted by deleting virulence-associated and host-range genes. In total, 18 genes were serially deleted from the genome to produce a virus which was designated the NYVAC strain. The attenuated nature of the resultant virus was demonstrated by the following criteria:

a) no detectable induration or ulceration at the inoculation site on rabbit skin

b) rapid clearance of infectious virus from the intradermal site of inoculation on rabbit skin

c) absence of testicular inflammation in nude mice

d) reduced virulence, as demonstrated by intracerebral inoculation into newborn mice

e) reduced ability to replicate in nude mice or immunosuppressed mice

f) reduced ability to replicate in human cells *in vitro* (46).

However, possible adverse reactions in humans have not yet been examined. A recombinant Aujeszky's disease vaccine expressing Aujeszky's disease virus glycoproteins was developed using this strain of vaccinia virus which, in contrast to the swinepox-recombinant mentioned in the previous section (see 'Recombinant vaccines based on mammalian poxvirus vectors', above) (50), gave significant protection when tested in pigs (10).

Recombinant vaccines based on avipoxvirus vectors

Live attenuated fowlpox vaccine has been widely used to produce recombinant vaccines for chickens. Fowlpox virus, an avipoxvirus, belongs to the same family as vaccinia virus (Chordopoxvirinae) and essentially the same approach can be used to produce the recombinant vaccines. Like the capripox-recombinant viruses described above, these fowlpox vaccines are dual vaccines, which can be used to protect chickens against both the disease specific to the inserted gene and also against virulent fowlpox. The other application of avipoxvirus vectors is their potential for use as highly safe vaccines for mammalian species, including humans, since

they do not produce infectious virus in non-avian cells. Although the avipoxviruses can efficiently infect mammalian cells, complete virus replication does not occur. The blockage probably occurs at the stage of late gene expression and no progeny virus is produced (43).

A recombinant vaccine against avian influenza, in which the HA gene of avian influenza virus was inserted into an attenuated fowlpox virus, was shown to protect chickens against challenge with virulent avian influenza virus (47). Other useful avian vaccines have been produced using the same fowlpox vector. Recombinant Newcastle disease virus (NDV) vaccine expressing the F protein gene was shown to confer protective immunity in chickens (48). A recombinant infectious bursal disease (IBD, or Gumboro disease) was also produced by inserting the VP2 gene of IBD virus into the fowlpox vector. This was shown to induce partial protective immunity in chickens against IBD (4). A second recombinant NDV vaccine expressing both the HN and F protein genes of NDV was licensed in the USA in 1994 and became the first commercially available vector vaccine (27).

A very active area of research is the application of avipoxvirus vectors for the production of recombinant vaccines for use in mammals. Although recombinant vaccines based on vaccinia virus vectors have been shown to be highly effective in generating protective immunity, vaccinia virus may cause adverse reactions in some people (see 'Further attenuation of the vaccinia virus vector', above). Although this risk is small, it has effectively delayed the use of recombinant vaccines, particularly as there are an increasing number of immunosuppressed people, due to HIV infection. A potential solution to this problem is the substitution of avipoxvirus as the vector (17). Despite the incomplete replication cycle, recombinant avipoxviruses expressing foreign genes have been shown to elicit protective immune responses in mammals. In preliminary experiments, two avipoxviruses, fowlpox and canarypox, were used as vectors to develop recombinant rabies vaccines expressing the G protein gene of the virus. By some as yet unknown mechanism, the canarypox vector induced a protective immunity against rabies more than 100 times higher, in terms of immunising dose, than that induced by the fowlpox vector. Canarypox-recombinant vaccines have been shown to confer protective immunity against rabies in mice, cats and dogs (17, 49), against viral haemorrhagic disease in rabbits (15) and against canine distemper in ferrets (44).

Recombinant vaccines based on herpesvirus vectors

Herpesviruses are also considered to be useful vectors for vaccines. The herpesvirus genome is a linear DNA molecule of approximately 150 kilobase pairs, into which foreign DNA can be inserted in a similar way to the treatment of poxviruses. However, due to the lack of suitably attenuated strains, attempts at developing vector vaccines have been limited. Live attenuated Aujeszky's disease virus expressing the envelope glycoprotein E1 of classical swine fever virus has been constructed, and was shown to protect pigs against both classical swine fever and Aujeszky's disease viruses (51).

Herpesvirus of turkeys (HVT) has been widely used as a live vaccine to protect against Marek's disease. A recombinant HVT was constructed by inserting either the fusion protein gene or the haemagglutinin-neuraminidase gene of NDV virus into a non-essential gene of HVT. Again, the resulting recombinant vaccine was shown to confer partial protective immunity against NDV in chickens, without affecting protective immunity against HVT (31). Feline herpesvirus (FHV) type 1 was used to develop recombinant vaccines against feline leukaemia virus (FeLV) and feline calicivirus infections. In the case of the feline calicivirus vaccine, the thymidine kinase gene was used for the insertion of the capsid protein gene. The recombinant vaccine was shown to induce neutralising antibody in cats following intranasal and oral immunisation, although protection against the virus challenge has not yet been tested (60). A recombinant vaccine expressing the glycoprotein gene of FeLV was reconstructed using FHV-1 as a vector. The weakly conserved gene (ORF 2) located downstream of the glycoprotein gC homologue in FHV was used as the insertion site for the FeLV glycoprotein gene, instead of the more usual thymidine kinase gene, which has been used in the case of the other alpha-herpesviruses. The recombinant vaccine was shown to protect cats from FeLV-induced viraemia by oronasal vaccination, indicating successful mucosal immunisation (52). Recently, the rabies virus G protein was expressed in canine herpesvirus and shown to protect dogs against challenge with the rabies virus (55).

Recombinant vaccines based on adenovirus vectors

Adenoviruses are being developed as gene delivery systems for gene therapy and, as a result, have also been investigated as vectors for the production of recombinant vaccines. An adenovirus-recombinant containing measles virus nucleoprotein gene has been shown to protect mice from virus challenge (16). Adenovirus has also been used as an alternative to poxvirus vectors for the production of recombinant vaccines for oral vaccination of wildlife against rabies (11, 58). The rabies virus G gene was inserted in place of the deleted E3 transcription unit of human adenovirus type 5, under the control of exogenous promoters or endogenous adenovirus promoters. Oral administration of the rabies adenovirus-recombinant vaccine protected skunks and foxes against rabies. Only transient faecal excretion of the virus was observed, indicating little or no replication of the virus in the intestine, and suggesting that minimal or no transmission would occur among animals under field conditions. The widespread presence of adenovirus type 5 in the human population was taken as evidence that this vector was safe for use in combating rabies in wildlife. However, the relatively heat-labile nature of adenovirus means that more

extensive studies on its environmental stability will be required if it is to replace the very effective poxvirus-recombinant vaccine for wildlife vaccination.

Recombinant vaccines based on positive-strand ribonucleic acid viruses

In the case of positive-strand RNA viruses, advances in molecular techniques have enabled scientists to modify individual genes, as in the case of DNA viruses. Moreover, because of their small size, whole virus genomes can be copied into DNA and manipulated at will. This not only allows known attenuating mutations to be incorporated into the genome to produce genetically defined and marked vaccines but also means that extra coding sequences for foreign proteins can be incorporated to produce vector vaccines. The RNA transcribed from a DNA clone *in vitro* is infectious if injected into a cell. To obtain infectious virus, injecting the DNA is sufficient since the RNA can be transcribed from this DNA in the cell, if it has a suitable promoter (38). This approach has been applied successfully to many plant and animal viruses (8).

Some unusual approaches to vaccination have been attempted using Picornaviridae, small positive-strand RNA viruses. The size and very ordered structure of these viruses do not allow insertion of significant amounts of foreign genetic material, but small pieces of DNA coding for known protective epitopes can be inserted in particular regions of the capsid proteins. Poliovirus is one of the safest and most widely used of the human vaccines, and DNA copies of its genome can be used to obtain live virus from transfected cells. In addition, the antigenic regions on the virus capsid protein have been identified and these have been replaced by protective epitopes from other viruses, most notably foot and mouth disease virus (FMDV) (25). The antigenic sites on FMDV capsid proteins have been extensively studied and mapped very accurately and, since the two viruses are similar in structure, it is possible to exchange antigenic sites without fatally disrupting the virus. These chimeric viruses have been shown to elicit immune responses specific to FMDV in guinea-pigs but have not been tested in the target animals for the vaccine: cattle, pigs, sheep and goats.

Other epitopes derived from HIV have also been inserted into the poliovirus genome. Plant picornaviruses, e.g. cowpea mosaic virus, have also had FMDV-protective epitopes inserted into their capsid proteins, with the hope that infecting plants with these virus chimeras will eventually lead to oral vaccination of the animals which eat them (37). The same authors also inserted epitopes from human rhinovirus 14 (HRV-14) and HIV into cowpea mosaic virus but only the HRV-14 chimera was tested for immunogenicity. There is no safely attenuated FMDV vaccine, and the protective response to purified inactivated virus antigen preparations is very short-lived. Vaccination, which is very costly, must be conducted every six months if it is to be effective, and so any improvement on the existing inactivated FMDV vaccines would be of great economic benefit. Such colourful approaches to vaccination may not, in the end, lead to useful vaccines, but they will provide insights into the immune responses to protective epitopes expressed in unusual ways.

Recombinant vaccines based on negative-strand ribonucleic acid viruses

The recent success in rescuing live negative-strand viruses from DNA copies of their genomes has made it possible to consider the use of these viruses as vectors for the delivery of foreign genes. Unlike the genomes of positive-strand RNA viruses, these genomes are non-infectious and the replicating unit is the negative-sense RNA genome, encapsidated by the nucleocapsid protein and associated with the polymerase and phosphoproteins which are needed to copy the messenger RNA. These accessory proteins must be provided in the cell together with the genome RNA to enable virus transcription and replication to occur (13). Since these viruses are pleiomorphic, there is less constraint on the size of the foreign DNA that can be inserted. The envelope glycoprotein 120 gene of HIV has been inserted into paramyxovirus genome (61).

The great advantage of such systems is the generally very long-lasting immunity generated following infection or vaccination with paramyxoviruses (e.g. measles and rinderpest viruses induce life-long immunity), and if such long-lasting immunity could be transferred to other diseases, such as FMD, the advantages would be considerable. A synthetic gene incorporating B- and T-cell stimulating epitopes from FMDV has been successfully inserted and expressed in the rinderpest virus genome and the protective immunity generated against FMDV is now being studied (2). The other advantage of this system is the ability to place marker genes into the genomes of paramyxovirus vaccine strains, with the consequent ability to distinguish vaccination from natural infection easily, using serological techniques (see 'Role of recombinant vaccines in vaccination campaigns', below).

Deoxyribonucleic acid vaccines

The idea of using naked DNA as a vaccine was proposed by Wolff and colleagues in 1990 when they found that genes such as chloramphenicol acetyltransferase, luciferase and beta-galactosidase were expressed when plasmid DNA

containing each gene was injected into the muscles of mice (53). Since then, DNA vaccines have been developed for a variety of diseases of both medical and veterinary importance.

A DNA vaccine consists only of *Escherichia coli*-derived plasmid DNA into which a DNA fragment coding for vaccine antigen is inserted. The vaccine gene is placed under the control of a strong mammalian promoter sequence and is taken up by and expressed in host cells when injected intramuscularly into animals. Thus, the DNA plasmid serves as an amplification system to produce sufficient DNA coding for the vaccine antigen gene for injection into the animal and is not an essential component of the vaccine. Unlike virus vectors, the plasmid is incapable of replicating in mammalian cells. Expression of the vaccine antigen under these conditions leads not only to the development of neutralising antibody but also to cytotoxic T lymphocytes (CTLs) in most cases. Owing to the simplicity of the manufacturing process and the inoculation procedure, and the efficiency with which both arms of the immune system are stimulated, DNA vaccination is now considered to be a highly promising approach to combating diseases against which more conventional vaccines have been ineffective.

Advantages of deoxyribonucleic acid vaccines

The production of DNA vaccines is based on the growth of bacterial plasmid DNA, so that large-scale production at high purity can be conducted inexpensively. As the bacterial plasmids apparently do not replicate in mammals, DNA vaccines are considered to be safe compared with the other live virus or bacterial vaccines, which may retain some pathogenic potential. In addition, since DNA is not absorbed by a receptor-dependent mechanism, there are no antibodies that can interfere with the uptake of DNA into the cell and it may be possible to immunise young animals with residual maternal antibody. As DNA vaccines consist only of plasmid DNA and should contain no contaminating proteins, multiple vaccinations would seem to be possible without generating immune responses to the vector DNA. The genetically stable nature of DNA would eliminate problems such as reversion to virulence of the vaccine, and it would be easier to construct DNA vaccines against pathogens which are either difficult to culture or dangerous to handle. Another advantage of DNA vaccines is the high stability of DNA preparations, which means that a cold chain for storage and transport of the vaccine is unnecessary.

Delivery of the DNA vaccine has been conducted in two basic ways, either by direct intramuscular injection or by particle bombardment. In the latter case, microscopic gold beads are coated with the vaccine DNA and shot into the skin cells using a special particle gun ('gene gun'), which has been widely used in the delivery of DNA constructs into plants to produce transgenic plants. The gene gun is a much more efficient means of delivery of the vaccine and greatly reduces the effective dose required. As these particle guns are very expensive at present, their veterinary application may have to await the development of more inexpensive apparatus.

One of the important features of DNA vaccines is their ability to induce a cell-mediated immune response through the production of CTLs, which are considered to be essential in protection against several virus infections (59). To induce CTL activity, the antigen must be produced in the cytoplasm of the host cell and then transported to the endoplasmic reticulum, where the antigen is processed and its peptides associated with a major histocompatibility complex (MHC) class I molecule, before being transported to the surface of the cell. There the peptide/MHC I complex comes into contact with the antigen-presenting cells. This contact results in the induction of CTLs which will recognise and kill cells expressing the complex on the surface. These antigen-processing and presentation processes apparently occur in muscle cells for antigens expressed from DNA vaccines. However, precise experimental evidence for this has not yet been reported.

Deoxyribonucleic acid vaccines for veterinary use

Owing to the advantages of DNA vaccines detailed above, they have been widely studied as candidate vaccines for both humans and animals and are considered to hold great potential for future vaccine development. Table II summarises the literature on DNA vaccines developed for veterinary use thus far. In most cases, the production of neutralising antibody as well as protection against virulent challenge has been shown to occur. In addition, CTL activity has been demonstrated for certain viruses, including bovine herpesvirus (14), rabies virus (54), influenza virus (18) and

Table II
Deoxyribonucleic acid vaccines under development

Vaccine	Antigen	Animals tested	Reference
Bovine herpes	Glycoprotein	Cattle, mice	(4)
Avian influenza	Haemagglutinin M protein Nucleoprotein	Chickens Ferrets Mice, monkeys	(18)
Lymphocytic choriomeningitis	Glycoprotein Nucleoprotein	Mice	(28)
Mycoplasma pulmonis	*M.p.* DNA *M.p.* DNA library	Mice	(3)
Rabies	Glycoprotein	Mice	(54)
Bovine viral diarrhoea	gp53(E2)	Mice	(20)
Feline immunodeficiency virus	Entire feline immunodeficiency virus genome	Cats	(39)
Newcastle disease	Fusion protein	Chickens	(40)
Aujeszky's disease	Glycoprotein D	Piglets	(30)

M : matrix
M.p. : *Mycoplasma pulmonis*
DNA : deoxyribonucleic acid
gp : glycoprotein

lymphocytic choriomeningitis (LCM) virus (59). Although the vaccine against LCM virus has no veterinary relevance, it can serve as a model for the effectiveness of DNA vaccines in cases of persistent viral infection (28).

Most DNA vaccines have been produced against virus diseases for which the protective antigens have been identified and well studied. A unique approach was used for *Mycoplasma pulmonis*, a mouse pathogen for which protective antigens have not yet been identified. Genomic DNA was cloned into a plasmid expression vector and the resulting library of gene fragments was used in mouse immunisation studies to screen for protective antigens. Protection was demonstrated in mice vaccinated with different expression libraries: so-called expression library immunisation. This method produces a multipartite vaccine which can be further screened to identify the essential protective epitopes of proteins. This approach may prove useful for pathogens which are difficult to grow or attenuate or in cases where the genes responsible for the protective immune response have not been identified (3).

Problems which remain to be solved

Although the effectiveness of DNA vaccine has been shown for a variety of viruses and other micro-organisms, some basic questions concerning the nature of the protection mechanisms remain unanswered. The DNA introduced into mouse muscle cells has been shown to persist episomally for up to 18 months. It is speculated that the DNA is continuously expressed and the antigen is processed for MHC class I presentation in the cytoplasm of muscle cells, but the type of cell in the muscle which presents the antigen for CTL induction is not known. Plasmid vectors expressing large amounts of gene product do not necessarily induce immune response to the encoded antigen. Short immunostimulatory DNA sequences, which contain a CpG dinucleotide in a particular base context, have been identified (41). These induce pro-inflammatory cytokines and improve the immune response to the plasmid-expressed foreign DNA.

Mechanisms to increase the immunogenicity of DNA vaccines are an important area for future study. A better understanding of the mechanisms that induce CTL activity will enhance the ability to design more effective conventional and DNA vaccines in future. As DNA vaccines rely on very strong viral promoters to drive expression of the foreign genes, accidental integration of DNA into the genome of those vaccinated is of major concern when contemplating use of these vaccines in humans. This kind of risk, however, may be trivial in most farm animals with short lifespans, but may have to be considered when using DNA vaccines for animals with long lifespans.

Role of recombinant vaccines in vaccination campaigns

In the future, recombinant vaccines may serve an additional purpose as marked vaccines. Since animals vaccinated with recombinant viruses can be distinguished serologically from naturally infected animals, the use of these vaccines will not interfere with serological studies to detect the presence of any wild virus type.

The potential usefulness of the recombinant vaccine approach was demonstrated in the case of avian influenza, as the US Department of Agriculture has prohibited the use of conventional vaccines in chickens because the vaccine complicates the serological screening programme to eliminate the disease. Rinderpest seromonitoring is currently based on the detection of antibody against the H protein (1). Animals which have been naturally infected will also have antibodies directed against the nucleoprotein (NP) of rinderpest virus, unlike the vaccinated animals. Testing sera using an enzyme-linked immunosorbent assay based on the presence of anti-NP antibodies will allow detection of continuing disease (23).

Countries wishing to declare freedom from rinderpest must follow the strict guidelines laid down by the Office International des Epizooties (OIE): the so-called 'OIE Pathway'. According to this protocol, provisional freedom from the disease can only be declared when mass vaccination has been ended and replaced by active serosurveillance to look for the presence of rinderpest virus (33).

In some areas of the world, the decision to end the use of conventional vaccine is often difficult, as neighbouring countries may still be infected and cessation of vaccination will result in a large number of susceptible animals. This might have devastating consequences if the disease is present but undetected. As a first step, recombinant vaccines could be used, as these will not interfere with serosurveillance. It would then be possible to detect hidden disease without the risk of creating a large population of susceptible animals.

Nouvelles méthodes de production de vaccins antiviraux à usage vétérinaire

K. Yamanouchi, T. Barrett & C. Kai

Résumé

Les progrès sensibles enregistrés depuis dix ans dans le domaine des technologies utilisant l'acide désoxyribonucléique (ADN) recombinant ont conduit à l'élaboration de plusieurs vecteurs de vaccins nouveaux et sûrs, capables d'exprimer efficacement des immunogènes étrangers. Ceux-ci, qui font appel à divers types de virus – poxvirus, herpèsvirus et adénovirus –, ont abouti à la production de nombreux vaccins recombinants potentiels. Parmi ces vaccins recombinants, le vaccin de la rage, dans lequel la protéine G de la rage s'exprime dans un vecteur de la vaccine, a été largement utilisé sur le terrain pour prévenir la propagation de cette maladie en Europe ainsi qu'aux États-Unis d'Amérique. Un vaccin à virus recombinant de la maladie de Newcastle, utilisant le virus de la variole aviaire comme vecteur pour exprimer des protéines immunogènes du virus, a été le premier vaccin commercial autorisé à vecteur recombinant. Nombre d'autres vaccins à virus recombinants sont actuellement à l'étude en laboratoire ou sur le terrain.

L'avancée la plus récente en vaccinologie a été la réussite de la vaccination avec de l'ADN nu. Cette méthode, qui semble très prometteuse sur souris, joue un rôle de premier plan dans le développement de nouveaux vaccins. Jusqu'à une période récente, la modification moléculaire des virus classiques à acide ribonucléique (ARN) en vue d'obtenir des vaccins à virus atténué plus stables n'était possible que pour les virus ARN à brin positif, tels que les poliovirus. Toutefois, les récents progrès accomplis par certaines techniques de biologie moléculaire ont permis de récupérer des virus à brin négatif à partir de répliques d'ADN de leurs génomes. On a ainsi pu procéder à des changements spécifiques dans les génomes des Rhabdoviridae et des Paramyxoviridae, qui incluent dans les deux cas plusieurs virus importants en médecine vétérinaire. Les auteurs décrivent dans cette étude le stade actuel du développement des vaccins à vecteur, des vaccins à ADN et des vaccins basés sur les génomes de virus ARN à brin positif et négatif obtenus par génie génétique, en mettant l'accent sur leur application à des maladies importantes en médecine vétérinaire.

Mots-clés

Adénovirus vecteur – Herpèsvirus vecteur – Poxvirus vecteur – Système de récupération – Vaccin à acide désoxyribonucléique – Vaccin à vecteur – Vaccin recombinant.

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Nuevas soluciones para el desarrollo de vacunas víricas de uso veterinario

K. Yamanouchi, T. Barrett & C. Kai

Resumen

El notable progreso que durante el pasado decenio experimentó la tecnología del ADN (ácido desoxirribonucleico) recombinante ha llevado a la obtención de un amplio surtido de vectores de vacuna nuevos y seguros, capaces de expresar con eficiencia inmunógenos ajenos. Pertenecientes a diversos tipos víricos –poxvirus, herpesvirus y adenovirus–, dichos vectores ofrecen la posibilidad de fabricar gran número de nuevas vacunas recombinantes. De ellas, la vacuna

contra el virus de la rabia, obtenida por expresión de la proteína G de este virus en un vector vaccinia, se ha utilizado de forma generalizada sobre el terreno, tanto en Europa como en Estados Unidos de América, para prevenir la propagación de la enfermedad. La primera vacuna recombinante vehiculada por un vector que obtuvo licencia de comercialización es una vacuna contra el virus de la enfermedad de Newcastle, que utiliza el virus de la viruela aviar como vector donde se expresan proteínas inmunógenas del primero. Hay muchas otras vacunas de virus recombinantes que se encuentran aún en fase de experimentación, ya sea en laboratorio o sobre el terreno.

La novedad más reciente en vacunología es el éxito obtenido con el uso de ADN desnudo como medio de vacunación. Este sistema ha deparado resultados prometedores en modelos con ratones, hasta convertirse hoy en día en el campo donde más activamente se trabaja para crear nuevas vacunas. Hasta hace poco tiempo, la modificación molecular de virus ARN (ácido ribonucleico) convencionales para obtener vacunas atenuadas más estables sólo era posible con virus ARN de cadena positiva, como los poliovirus. Sin embargo, recientes progresos en las técnicas de biología molecular hacen posible rescatar virus de cadena negativa a partir de copias ADN de su genoma. Ello, a su vez, ha permitido introducir cambios específicos en el genoma de Rhabdoviridae y Paramyxoviridae, familias ambas que incluyen diversos virus de importancia veterinaria. Los autores describen los últimos avances en la fabricación de vacunas vehiculadas por vectores, vacunas de ADN y vacunas basadas en la modificación del genoma de virus ARN de cadena positiva o negativa, haciendo especial hincapié en sus aplicaciones a enfermedades de importancia veterinaria.

Palabras clave

Sistema de rescate – Vacuna de ácido desoxirribonucleico – Vacuna recombinante – Vacuna vehiculada por un vector – Vector adenovirus – Vector herpesvirus – Vector poxvirus.

■

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Clinical studies of gene therapy for cystic fibrosis (CF) suggest that the key problem is the efficiency of gene transfer to the airway epithelium. The availability of relevant v ctor receptors, the transient contact time between vector and function of airway mucus contribute

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Viral gene delivery to motor neurons

Efficient gene transfer to airway epithelium using recombinant Sendai virus

Yoshikazu Yonemitsu[1], Christopher Kitson[1], Stefano Ferrari[1], Raymond Farley[1], Uta Griesbach[1], Diane Judd[1], Rachel Steel[1], Philippe Scheid[1], Jie Zhu[1], Peter K. Jeffery[1], Atsushi Kato[2], Mohammad K. Hasan[2], Yoshiyuki Nagai[3], Ichiro Masaki[4], Masayuki Fukumura[5], Mamoru Hasegawa[5], Duncan M. Geddes[1], and Eric W.F.W. Alton[1]*

[1]*Department of Gene Therapy, Imperial College School of Medicine at the National Heart and Lung Institute, London, UK.* [2]*Department of Viral Infection and Vaccine Control, National Institute for Infectious Diseases, Tokyo, Japan.* [3]*AIDS Research Centre, National Institute for Infectious Diseases, Tokyo, Japan* [4]*Division of Pathophysiological and Experimental Pathology, Department of Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan.* [5]*DNAVEC Research Inc., Tsukuba-city, Ibaraki, Japan.* *Corresponding author (e.alton@ic.ac.uk).

Clinical studies of gene therapy for cystic fibrosis (CF) suggest that the key problem is the efficiency of gene transfer to the airway epithelium. The availability of relevant vector receptors, the transient contact time between vector and epithelium, and the barrier function of airway mucus contribute significantly to this problem. We have recently developed recombinant Sendai virus (SeV) as a new gene transfer ag nt. Here we show that SeV produces efficient transfection throughout the respiratory tract of both mice and ferrets in vivo, as well as in freshly obtained human nasal epithelial cells in vitro. Gene transfer efficiency was several log orders greater than with cationic liposomes or adenovirus. Even very brief contact tim was sufficient to produce this effect, and levels of expression were not significantly reduced by airway mucus. Our investigations suggest that SeV may provide a useful new vector for airway gene transfer.

Keywords: Sendai virus, cystic fibrosis, gene therapy

Clinical trials of gene therapy for cystic fibrosis (CF) have demonstrated encouraging steady progress, indicating that transfer of the cystic fibrosis transmembrane conductance regulator (CFTR) gene can partially correct the chloride transport defect in human subjects[1–5]. Despite this progress, current levels of gene transfer efficiency are probably too low to result in clinical benefit—in large part as a result of the barriers faced by gene transfer vectors within the airways.

The respiratory epithelium has evolved a complex series of extracellular barriers aimed at preventing penetration of lumenally delivered materials into either the cell or the interstitial compartment. In series, these comprise a mucus layer that may bind inhaled vectors and clear them by mucociliary clearance mechanisms, a glycocalyx that may prevent binding to cell surface receptors, and an apical cell membrane that is relatively devoid of both viral receptors (such as those for adenovirus and adeno-associated virus) and those for synthetic vectors. In addition, the apical surface of well-differentiated airway epithelial cells has a low basal and stimulated rate of endocytosis, thus discouraging vector entry. Basolateral access is potentially more promising because the relevant receptors are located on this surface, but access to this region is limited by tight junctions. Even once the DNA has overcome the extracellular barriers, a number of intracellular barriers follow, including endosomal escape, cytoplasmic trafficking and passage through the nuclear pore complex.

Two approaches to solving these problems are possible. Either new vectors must be devised that can overcome this formidable series of barriers, or pharmacological manipulation of the barriers themselves can be attempted. We[6] and others[7] have described ways to undertake the latter, but improvements in gene transfer efficiency have been insufficient to warrant clinical trials with these agents. Recombinant Sendai virus (SeV), a member of the paramyxoviridae[8,9], is a single-stranded RNA virus that we have demonstrated to produce efficient gene transfer and expression in vitro[9]. We have now assessed the use of SeV for airway gene transfer in vivo.

Results and discussion

In vivo gene transfer to airway epithelial cells of mice and ferrets. First, we evaluated in vivo gene transfer in the murine lung, a commonly used model. Two days after administration of SeV encoding luciferase (SeV-luc) to the lungs, there was a dose-dependent increase in expression (Fig. 1), with values approximately 3–4 logs higher than those obtained with 80 μg pCMV-luc complexed to the cationic lipid GL-67 (ref. 6). The rodent lung is the natural host of the wild-type SeV strain that was used to construct the recombinant virus[10] and may, therefore, represent a uniquely permissive host. To exclude this we assessed gene transfer efficiency in the ferret lung in vivo. The lung morphology, mucin composition, and density of submucosal glands of these animals are similar to those in humans[11], providing a more relevant model than does the murine lung. We administered SeV encoding nuclear-localized β-galactosidase (SeV-LacZ) to allow histological assessment of gene transfer efficiency. Dose-dependent β-galactosidase expression was seen throughout the conducting airways (Fig. 2A–F). Approximately 20–30% of epithelial cells expressed β-galactosidase when infected with 3×10^8 p.f.u., and 70–80% of cells when 3×10^9 p.f.u. were administered (Fig. 2G). No β-galactosidase expression was seen in any of the control samples. In keeping with the reported tropism of SeV (ref. 12), X-Gal-positive cells included both ciliated columnar and nonciliated secretory cells but no apparent signal was detected in basal cells. Interestingly, β-galactosidase-expressing cells were also found in submucosal glands (Fig. 2E), the predominant site of CFTR expression in the human bronchus[13], ~1–3% of gland cells were positive. Ferret conducting airways and parenchyma were assessed for the presence of inflammation by semi-quantitative analysis. The degree of inflammation varied from little or none in the left upper lobe to mild to moderate in the most dependent right lower lobes for a given SeV titer. The infiltrate was typically composed of monocytes, lymphoid cells, and, to a lesser extent, neutrophils, and was dose dependent. This pattern of inflammation was predominantly restricted to third-generation airways and smaller. At the higher viral

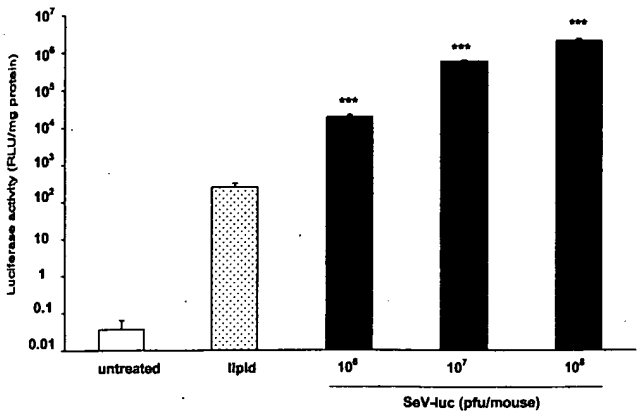

Figure 1. Comparison of SeV and liposome-mediated gene transfer to the mouse lung in vivo. Both vectors, encoding luciferase, were applied to the nose in a single bolus (100 μl) and rapidly sniffed into the lung (n = 5–10 for each group; error bars indicate s.e.m.; ***$P <$ 0.001 compared to cationic lipid). Note log scale.

titer, a neutrophil-dominated airway lumen exudate was also seen.

In vitro gene transfer to freshly obtained human airway epithelium. To assess whether human airway epithelial cells are susceptible to SeV infection, we assessed SeV-mediated gene transfer to freshly obtained primary human nasal epithelium in vitro. As in the above studies, SeV-luc produced efficient gene transfer, which was dose dependent, and up to 3 logs higher than for lipid (Fig. 3). Laboratory studies suggest that correction of the CF chloride defect can be achieved by introducing ~6–10% of corrected cells overexpressing CFTR into a CF monolayer[14]. Even if this is an underestimate, the demonstrated transfection efficiency of SeV in the tested models appears to be sufficient to allow correction of the CF chloride defect.

Dependence of SeV-mediated gene transfer on its sialic acid receptor. To demonstrate SeV specificity for the sialic acid receptor, UV-inactivated SeV encoding green fluorscent protein (SeV-GFP) was added for 1 h to MDCK cells grown to confluence to allow formation of tight junctions. The hemagglutinin neuraminidase (HN) protein of UV-inactivated SeV retains neuraminidase activity, producing removal of sialic acid residues from the receptor. The MDCK cells were then rechallenged with SeV-luc 24 h later. Expression was significantly reduced from $3.1 \times 10^9 \pm 1.8 \times 10^8$ Relative Light Units(RLU)/mg protein (untreated cells) to $8.5 \times 10^7 \pm 8.7 \times 10^6$ RLU/mg protein (following addition of UV-inactivated SeV, $P < 0.01$). Restoration of transgene expression to values not significantly different from those obtained with untreated cells was obtained 14 days later ($2.7 \times 10^9 \pm 1.5 \times 10^8$ RLU/mg protein).

Effect of contact time on SeV-mediated gene transfer. Both cationic lipid[6] and adenovirus-mediated[15] gene transfer are dependent on prolonged contact time with the target epithelium. However, in humans mucociliary clearance and the presence of macrophages provide efficient clearance mechanisms within the lung, limiting such contact time. In vitro studies showed that Cos7 cells exposed to SeV-luc for 1 min have only 2.5-fold lower luciferase expression than that obtained from cells exposed for 24 h to SeV. Further, <10% of the

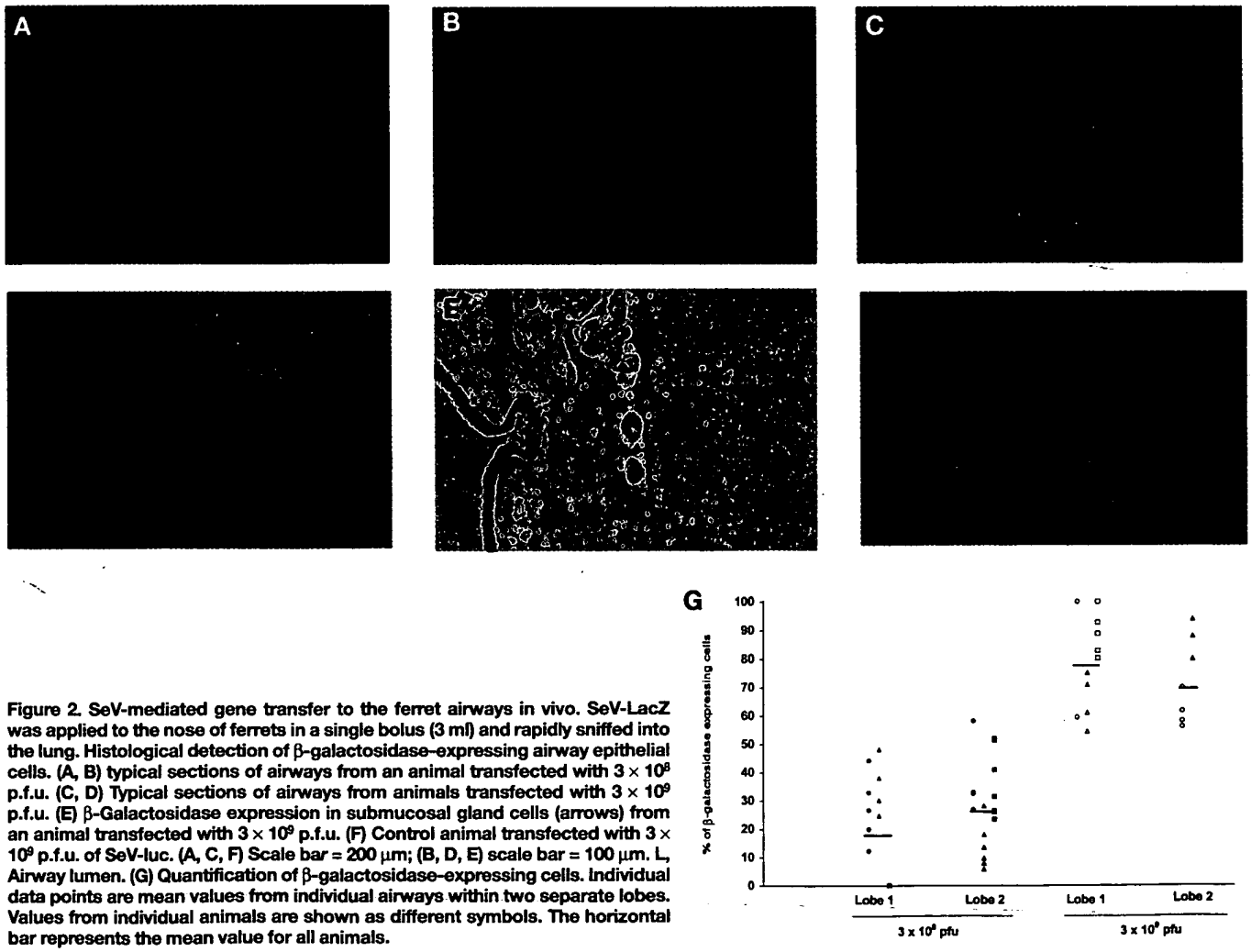

Figure 2. SeV-mediated gene transfer to the ferret airways in vivo. SeV-LacZ was applied to the nose of ferrets in a single bolus (3 ml) and rapidly sniffed into the lung. Histological detection of β-galactosidase-expressing airway epithelial cells. (A, B) typical sections of airways from an animal transfected with 3×10^8 p.f.u. (C, D) Typical sections of airways from animals transfected with 3×10^9 p.f.u. (E) β-Galactosidase expression in submucosal gland cells (arrows) from an animal transfected with 3×10^9 p.f.u. (F) Control animal transfected with 3×10^9 p.f.u. of SeV-luc. (A, C, F) Scale bar = 200 μm; (B, D, E) scale bar = 100 μm. L, Airway lumen. (G) Quantification of β-galactosidase-expressing cells. Individual data points are mean values from individual airways within two separate lobes. Values from individual animals are shown as different symbols. The horizontal bar represents the mean value for all animals.

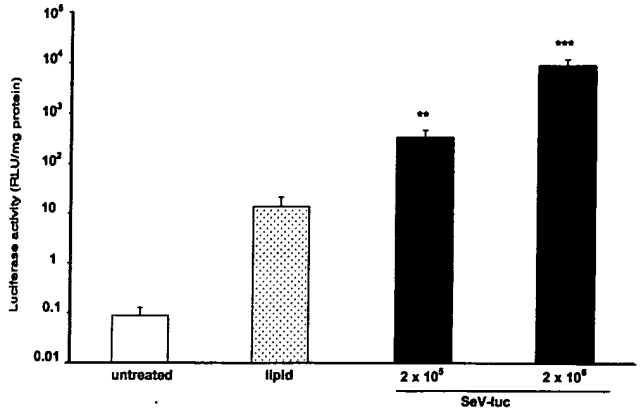

Figure 3. Gene transfer to freshly obtained human nasal epithelium ex vivo. Cells were transfected with either SeV or plasmid–lipid complexes encoding luciferase and assessed 24 h following gene transfer (n = 5–16 for each group, where n refers to subject number; error bars indicate s.e.m.; **$P < 0.01$ and ***$P < 0.001$ compared to cationic lipid). Note log scale.

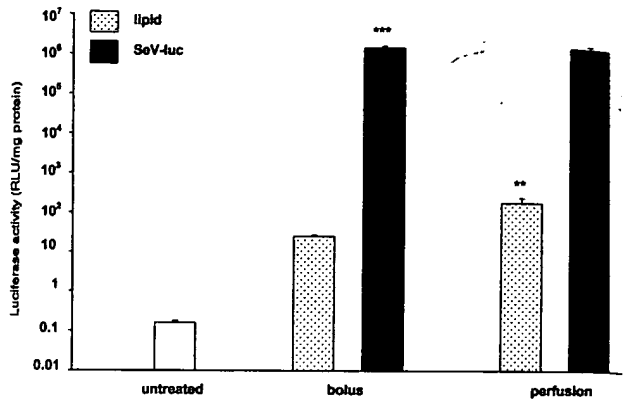

Figure 4. Effect of contact time on gene expression in the mouse nose in vivo. Plasmid complexed to lipid or SeV (10^7 p.f.u./ml), both encoding luciferase, was applied to the nose either as a single 100 µl bolus or by prolonged perfusion (n = 5–10 in each group; error bars indicate s.e.m.; **$P < 0.01$ compared with respective bolus; ***$P < 0.001$ compared with lipid bolus).

SeV-luc could be recovered from the culture medium 5 min after addition to the cells. This suggests either that there is rapid uptake of SeV into cells or that SeV remains adherent to the cell surface, providing a reservoir for more prolonged internalization. To assess whether a short duration of exposure is sufficient to induce expression in the airways in vivo, we administered SeV-luc to the murine nasal epithelium. This is a well-characterized model for airway gene transfer that allows changes in vector contact time to be assessed in vivo, either by placement of a single bolus within the nasal cavity (cleared within 30 s by sniffing) or by continuous perfusion over ~30 min. In either case, transgene expression was assayed in the nasal epithelium 48 h later. Following the bolus application, SeV produced a >4 log increase in transgene expression in comparison to lipid (Fig. 4). No further increase in expression was seen following prolonged perfusion, suggesting rapid uptake of SeV into the respiratory epithelium in vivo. As expected, lipid-mediated gene expression was significantly increased by prolonged contact time, but levels remained ~4 logs lower than achieved by SeV. Thus, SeV achieves gene transfer following only brief contact with the airway epithelium, as is likely to be the case in the human airway in vivo.

Effect of the airway mucus barrier on SeV-mediated gene transfer. We have recently shown that native mucus inhibits lipid-mediated gene transfer[6]. To evaluate the effect of mucus as a barrier on SeV-mediated gene transfer, we used a previously described sheep tracheal model, in which fresh tissue is maintained at an air/liquid interface. The model retains extracellular barriers to gene transfer such as mucus coverage and mucociliary clearance[6]. Furthermore, it is characterized by intact pseudostratified columnar epithelium, including tight junctions, and likely has similar intracellular barriers to the native tissue. Lipid-mediated gene transfer was reduced more than 13-fold in the presence of mucus as already reported. In contrast, SeV produced 3–4 logs higher expression than for the lipid; this was only reduced twofold in the presence of mucus (Fig. 5). Therefore, mucus is not an important barrier for SeV-mediated gene transfer.

Comparison between Sendai and adenoviral vectors. We compared SeV and adenovirus-mediated gene transfer in the murine nose in vivo. This site was chosen because it closely resembles the morphology of its human counterpart. In contrast, the murine conducting airways are predominantly composed of Clara cells[16], previously shown to be highly susceptible to adenoviral gene transfer, but which are not abundant in human airways.

Using a single bolus application of an equivalent titer of either virus (10^7 p.f.u.), SeV-luc produced dramatically higher levels of luciferase expression (1,675,774 ± 91,192 RLU/mg protein, n = 8) than Adeno-luc (647 ± 291 RLU/mg protein, n = 8), in keeping with the above studies. Finally, we also compared the two viral vectors (10^7 p.f.u.) in the sheep ex vivo model, which also allows comparison of transfection efficiency in intact and damaged epithelium, the latter occurring at the cut edges of the preparation. This is of relevance for airway gene transfer, since adenovirus has been shown (in the absence of Clara cells) only to transfect damaged airway epithelium. In keeping with published data, Adeno-luc produced very low levels of transfection in intact sheep airways (16 ± 4 RLU/mg protein), but this increased significantly ($P < 0.001$) in the damaged edge portions (435 ± 104 RLU/mg protein). In contrast SeV-luc produced higher levels of expression in intact (43,434 ± 8,275 RLU/mg protein) and edge segments (90,957 ± 13,805 RLU/mg protein, n = 12–18 for each group). Thus, SeV produces 3–4 logs higher transfection efficiency than adenovirus in the respiratory epithelium and does not require damaged epithelium to achieve this.

Advantages of the new Sendai virus vector for airway gene transfer. There are several reasons why SeV may be highly effective for airway epithelial gene transfer. The receptor for SeV is sialic acid bound to gangliosides, present on most cell types, including the apical surface of the conducting airways[17]. Thus, in contrast to other new viral vectors currently under development (e.g., lentiviruses and adeno-

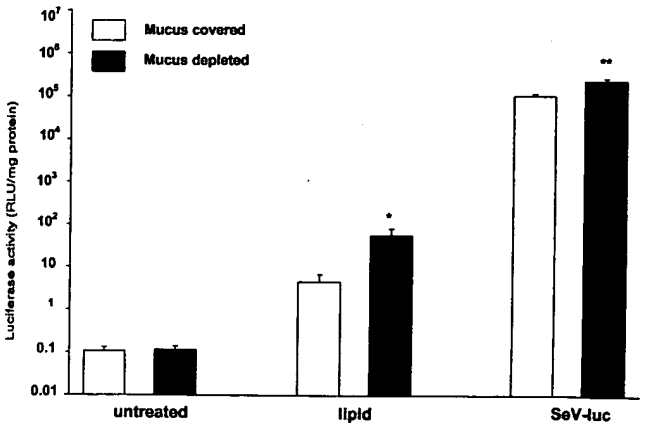

Figure 5. Gene transfer to mucus-covered and mucus-depleted sheep tracheae ex vivo. Tracheae were either maintained in their native mucus-covered state or were depleted of mucus before transfection (n = 9–24 in each group; error bars indicate s.e.m.; *$P < 0.05$ and **$P < 0.01$ compared to respective mucus-covered tissues). Note log scale.

associated virus), there is no need to access the basolateral surface of the airways through tight-junction modulation or to retarget the natural tropism of the virus. Additionally, as we show in this study, entry into the cell is not significantly limited by surface mucus, and occurs within a few minutes. A combination of receptor availability, mucus, and a need for longer contact time are important limiting factors in current liposome- and adenoviral-mediated airway gene transfer. Finally, SeV mediates gene transfer to a cytoplasmic location[18], in contrast to the endosomal uptake of current vectors. Furthermore, exogenous gene expression occurs at this site, removing the additional barriers of nuclear import. Thus, the efficiency of SeV-mediated gene transfer is consistent with current views on the key barriers to airway gene transfer, and may allow respiratory gene therapy to pass through the current bottleneck of inefficient gene transfer.

Experimental protocol

Viral and nonviral vectors. Recombinant SeVs were constructed and propagated as previously described[8,9,19,20]. Viral titers were determined by a standard chicken red blood cell hemagglutination assay and plaque assay on CV1 cells, and viruses were stored at –80°C. Ad-luc is a serotype 5 adenovirus encoding the firefly luciferase gene driven by the cytomegalovirus immediate early (CMV-IE) promoter. Adenovirus titer was determined by plaque assay on 293 cells to allow comparison of active viral particles. The cationic liposome GL-67/DOPE/DMPE-PEG$_{5000}$ was kindly provided by Dr S.H. Cheng (Genzyme Corp., Framingham, MA).

Gene transfer in murine airways. Recombinant SeV or adenovirus encoding luciferase[21], or the plasmid pCMV-luc complexed to GL-67/DOPE/DMPE-PEG$_{5000}$ (80 μg DNA/mouse) were placed as a single 100 μl bolus into the nasal cavity of male BALB/c mice (six to eight weeks), and the solution was sniffed into the lungs[22]. For nasal perfusion, a fine-tip catheter was placed 5 mm within the nasal cavity and 150 μl of the appropriate vector solution perfused at a rate of 5 μl/min using a peristaltic pump. Two days after gene transfer, nasal turbinates and lungs were harvested and assayed for luciferase activity[23].

Sendai virus–mediated gene transfer to ferret lungs. Ferrets (500–600 g weight) were similarly instilled with a single 3 ml bolus of various concentrations of SeV-LacZ (nuclear localized) or the control SeV-luc. Two days post infection, ferrets were killed and the lungs fixed by intratracheal perfusion of fixative solution, excised en bloc, processed, and stained with X-Gal as described by Ferrari and colleagues[24]. Each lung was sectioned and β-galactosidase-expressing cells quantified microscopically by point counting the airway epithelia and submucosal glands in tissue sections of two lobes. For each airway, 10 microscopic fields were sampled to obtain the percentage of β-galactosidase-expressing cells per airway, and three to eight airways, randomly taken from different regions of each lobe, were assessed. For submucosal glands, 10–28 fields (containing at least four glands per lobe) were assessed. The error of repeat measurement expressed as a coefficient of variation (CV) was 18%.

In vitro SeV-mediated gene transfer. Nasal epithelial cells, collected from healthy human donors (six male, three female) by nasal brushing, were transfected as described by Stern and colleagues[25]. SeV-luc was used at 2×10^5 and 2×10^6 p.f.u. The viability of the nasal cells was confirmed by phase-contrast microscopic evaluation of ciliary beating, as well as trypan blue exclusion. The mean recovery was 4.5×10^5 cells per sample (range 2.0 to 8.1×10^5), with a mean percentage of living cells of 38.4% (range 10–59%). Luciferase expression was measured 24 h after gene transfer. Note that the basolateral surface of these cells is also accessible to SeV in this assay.

MDCK cells seeded at 10^6 cells/well were grown to confluence (estimated cell number: 1 to 3×10^7 cells/well) and treated for 1 h with UV-inactivated (2000 mJ/cm^2) SeV-GFP (multiplicity of infection, MOI = 100). Cells were washed and infected with SeV-luc (MOI = 100) at appropriate time points. Luciferase activity was measured 48 h later. Cos7 cells were seeded at 10^5 cells/well and infected with SeV-luc (MOI = 10) at appropriate time points. Luciferase activity was assayed 48 h later.

Ex vivo gene transfer to sheep trachea epithelium. The ex vivo sheep trachea model was set up and transfected as described by Kitson and colleagues[6]. For lipid-mediated transfection, GL-67/DOPE/DMPE-PEG$_{5000}$ was complexed with pCMV-luc in a 1:4 ratio (molarity of lipid:nucleotide concentration) such that 5 μl of complex contained 12.5 μg plasmid DNA. SeV-luc was used at 10^8 p.f.u./tissue. Both fresh and mucus-depleted tissues were then incubated for 48 h before being assessed for luciferase activity.

Statistical analysis. All values are expressed as the mean ± s.e.m. for convenience, and n refers to the number of subject or animal number. The data were analyzed by the Mann–Whitney U-test, and the null hypothesis rejected at $P < 0.05$.

Ackn wl dgm nts

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virus P and NP proteins that are involved in the interaction with the L protein.

AUTHOR(S): Nishio, Machiko (1); Tsurudome, Masato; Ito, Morihiro; Ito, Yasuhiko

CORPORATE SOURCE: (1) Department of Microbiology, Mie University School of Medicine, 2-174, Edobashi, Tsu-Shi, Mie-Ken, 514-8507 Japan

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AB Eleven monoclonal antibodies (MAbs) directed against the large (L) protein of human parainfluenza type 2 virus (hPIV-2) were prepared to examine the interactions of the L protein with other viral proteins. Coimmunoprecipitation assays using these MAbs revealed that the L protein directly interacted with the phospho- (P) and nucleocapsid (NP) proteins in vivo and in vitro. Mutational analysis of the P or NP protein was performed to identify the region(s) on these proteins interacting with L protein, indicating that amino acids 278-353 on the P protein and amino acids 403-494 on the NP protein are essential for the binding to the L protein.

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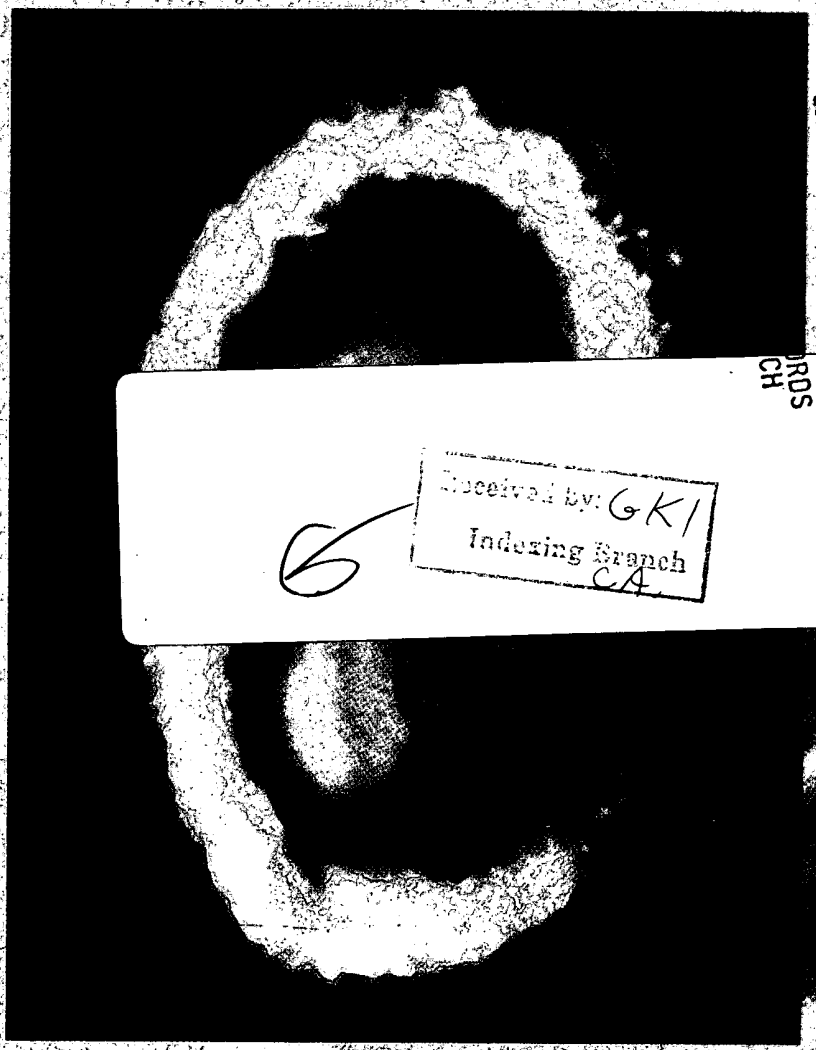

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Mapping of Domains on the Human Parainfluenza Type 2 Virus P and NP Proteins That Are Involved in the Interaction with the L Protein

Machiko Nishio,[1] Masato Tsurudome, Morihiro Ito, and Yasuhiko Ito

Department of Microbiology, Mie University School of Medicine, 2-174, Edobashi, Tsu-Shi, Mie-Ken 514-8507, Japan

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Eleven monoclonal antibodies (MAbs) directed against the large (L) protein of human parainfluenza type 2 virus (hPIV-2) were prepared to examine the interactions of the L protein with other viral proteins. Coimmunoprecipitation assays using these MAbs revealed that the L protein directly interacted with the phospho- (P) and nucleocapsid (NP) proteins *in vivo* and *in vitro*. Mutational analysis of the P or NP protein was performed to identify the region(s) on these proteins interacting with the L protein, indicating that amino acids 278–353 on the P protein and amino acids 403–494 on the NP protein are essential for the binding to the L protein. © 2000 Academic Press

INTRODUCTION

Human parainfluenza type 2 virus (hPIV-2) is one of the major human respiratory pathogens and a member of the genus *Rubulavirus* in the family *Paramyxoviridae*, nonsegmented and negative-stranded RNA viruses. Its RNA genome is approximately 15 kb in length and, like other paramyxoviruses, encodes seven structural proteins: the nucleocapsid (NP), phospho- (P), V, matrix (M), hemagglutinin-neuraminidase (HN), fusion (F), and large (L) proteins. The P and L proteins of paramyxoviruses are associated with the nucleocapsid and function as the RNA-dependent RNA polymerase. Although the three RNA-associated proteins thus play important roles in the replication cycle of the virus, the functional domains that participate in the protein–protein interactions among these three polypeptides have yet to be defined completely. In the case of Sendai virus, the formation of NP–P and P–L protein complexes is essential for nucleocapsid RNA replication, and the P and L RNA polymerase subunits must be coexpressed in the same cell to form a functional polymerase (Horikami *et al.*, 1992). The L protein alone is unable to bind to nucleocapsids, and its binding to the polymerase complex occurs through a P–nucleocapsid interaction (Ryan and Portner, 1990; Horikami and Moyer, 1995).

In our previous studies, we isolated a large number of monoclonal antibodies (MAbs) directed against the P or NP protein of hPIV-2 (Tsurudome *et al.*, 1989), mapped the epitopes recognized by these MAbs, and identified the regions essential for NP–P, NP–NP, and P–P interactions (Nishio *et al.*, 1996, 1997, 1999a). However, as compared with information about the functional domains on the P and NP proteins, there is less known about the structure–function relationships of the L protein. One of the reasons is that no MAb directed against the L protein of hPIV-2 is available. Here, for the first time, we have prepared anti-hPIV-2 L protein MAbs, which were obtained by immunizing mice with the bacterially expressed polypeptide representing the N-terminus of the hPIV-2 L protein. By Western blot assay using these MAbs, we have shown that the L protein directly interacts with the P and NP proteins *in vivo* and *in vitro*. Furthermore, mutational analysis of the P or NP protein was performed to identify the region of the P or NP protein interacting with the L protein.

RESULTS

Production of MAbs specific for hPIV-2 L

To obtain MAbs specific for the L protein, a polypeptide corresponding to the L protein N-terminal 374 amino acids was expressed in *Escherichia coli BL21 (DE3)*, purified, and used as the immunogen. Hybridomas were screened for their secreting antibody by ELISA using the plates that were coated with the bacterially expressed protein. Eleven hybridoma clones secreting antibodies that specifically reacted with the N-terminal polypeptide were obtained. The specificities of the L-specific MAbs were confirmed by Western blotting. Figure 1A shows the reactivity of MAbs L10-6, L60-2, and L70-6, three of the eleven MAbs, with purified SV41 (Fig. 1A, lane 1) or hPIV-2 (Fig. 1A, lane 2) virions. The MAbs reacted exclusively with hPIV-2 L polypeptide of approximately 220K, but did not react with the L protein of SV41.

Using the MAbs obtained in this study, we investigated the distribution of the L protein in hPIV-2-infected cells. Vero

[1] To whom correspondence and reprint requests should be addressed. Fax: +81-59-231-5008. E-mail: nishio@doc.medic.mie-u.ac.jp.

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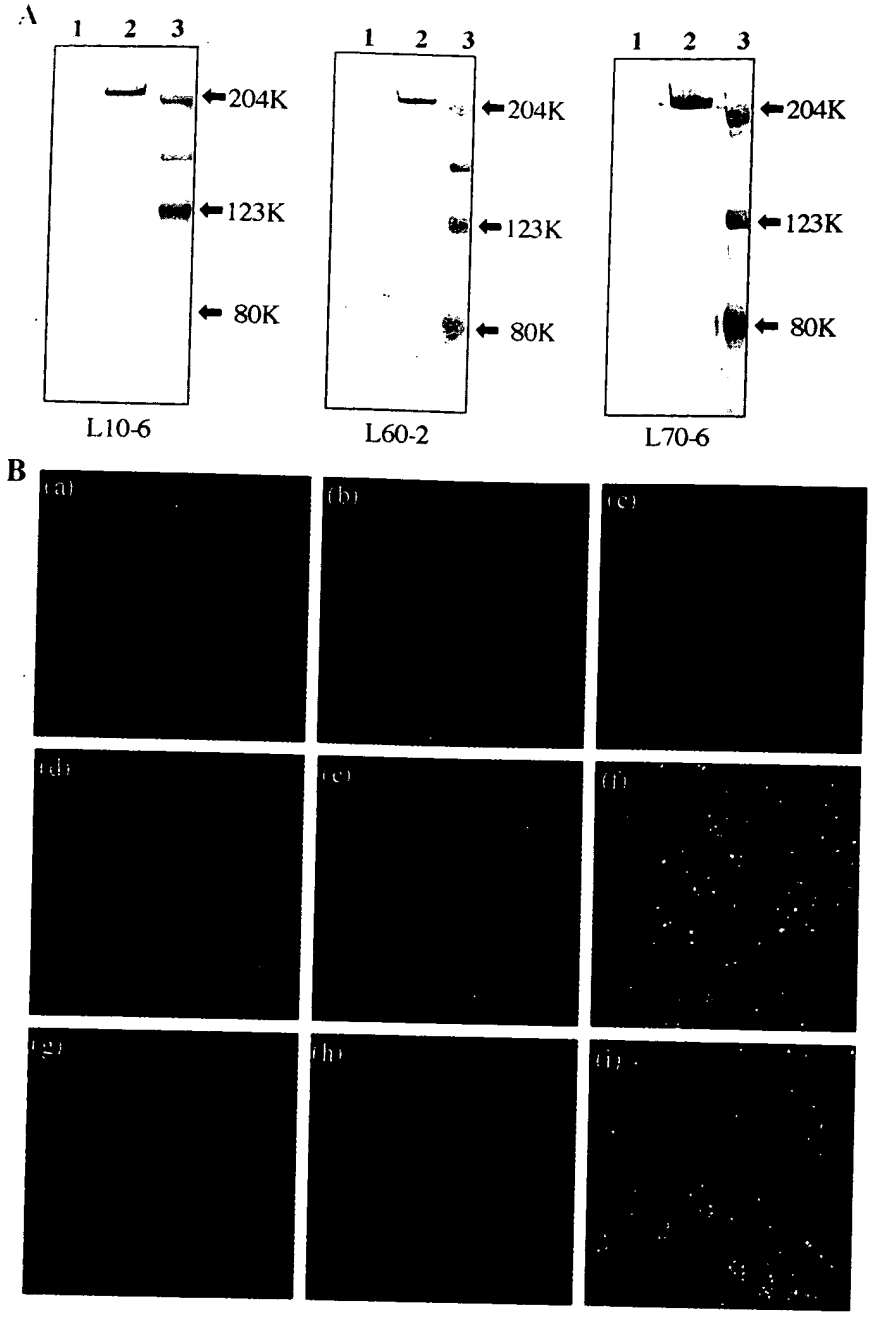

FIG. 1. Reactivity of MAbs in Western blot assay and immunofluorescent staining. (A) Purified SV41 (lane 1) and hPIV-2 (lane 2) virions were subjected to 9% LDS–PAGE. The proteins were transferred to a nitrocellulose membrane and probed with MAbs L10-6, L60-2, and L70-6. Lane 3, molecular weight markers. (B) Vero cells were infected with hPIV-2 at m.o.i. of 5. After 18 h, the infected cells and uninfected cells were fixed with 10% paraformaldehyde for 15 min and permeabilized with 0.05% Triton X-100 in PBS. The cells were treated with anti-L MAb (L10-6) [red: (c), (e), (f), (h), (i)] and anti-P MAb (61-2A) [green: (a), (d), (f)] or anti-NP MAb (20A) [green: (b), (g), (i)]. After being washed with PBS, the cells were treated with FITC-conjugated anti-mouse IgG1 goat serum and rhodamine-conjugated anti-mouse IgG2a goat serum.

cells were infected with hPIV-2, fixed at 18 h postinfection (p.i.) and then double-immunostained with anti-L MAb L10-6 and anti-P MAb 61-2A or anti-NP MAb 20A. These MAbs showed negative staining on uninfected control cells [Fig. 1B(a)–(c)]. The L protein showed diffuse staining pattern throughout the cytoplasm and formed fewer granules [Fig. 1B(e) and (h)] than did the P or NP protein [Fig. 1B(d) or (g)]. However, some of the L protein colocalized with the P or NP protein [Fig. 1B(f) or (i)].

Interaction between the L and P proteins

To examine the direct interaction between the L and P proteins of hPIV-2, HeLa-CD4^{+} cells were infected with

VVT7 and transfected with both the plasmid encoding L (pCR-L) and the plasmid encoding P (pCR-P). The cell lysates were immunoprecipitated with anti-L MAbs (mixture of L70-6 and L60-2) or anti-P MAbs (mixture of 85A and 335A), and then the immunoprecipitates were analyzed by the Western blot assay. As shown in Fig. 2A(a), the P–L complex is detected in the immunoprecipitates obtained with either anti-P or anti-L antibodies [Fig. 2A(a), lanes 1 and 2]. When the L and P proteins were synthesized separately and cell extracts were then mixed, the P–L complexes formed [Fig. 2A(b), lanes 5 and 6]. These coimmunoprecipitations were the result of specific precipitation of either the P or L protein, because under the same precipitation conditions, the anti-L antibodies did not precipitate the P protein in the absence of the L protein [Fig. 2A(b), lane 1]; similarly, the anti-P antibodies did not precipitate the L protein in the absence of the P protein [Fig. 2A(b), lane 4]. Furthermore, we tested whether such complex could be formed in a mixture of the L protein expressed in HeLa-CD4$^+$ cells and purified P protein expressed in the *E. coli.* Neither anti-P nor anti-L MAbs immunoprecipitated P–L complex [Fig. 2A(c), lanes 5 and 6], though the recombinantly expressed P protein was well reacted with anti-P MAbs [Fig. 2A(c), lane 2].

To identify domains on the P protein required for binding to the L protein, we used a set of plasmids encoding deleted P proteins [Fig. 2B(a)]. Each deleted P protein or the L protein was synthesized separately in HeLa-CD4$^+$ cells and cell extracts were then mixed. The ability to form complexes was tested in coimmunoprecipitation assays with anti-P MAb, 85A, or anti-L MAb, L70-6 [Fig. 2B(b) and (c)]. The complex $P_{\Delta C39}$–L was detected in the immunoprecipitates obtained with either anti-P or anti-L antibody [Fig. 2B(b), lanes 5 and 6], but complex $P_{\Delta C118}$–L could not be detected [Fig. 2B(c), lanes 3 and 4]. To further identify the domain essential for P–L complex, the L protein and the internally deleted P protein were tested [Fig. 2B(d)]. Although the internally deleted protein $P_{\Delta 272-353}$ was clearly expressed in transfected cells [Fig. 2B(d), lane 2], complex $P_{\Delta 272-353}$–L was not precipitated by either anti-P or anti-L MAbs [Fig. 2B(d), lanes 3 and 4]. These results suggest that region of amino acids 278–353 of the P protein is critical for interaction with the L protein.

Interaction between the L and NP proteins

Likewise, to examine the direct interaction between the L and NP proteins, HeLa-CD4$^+$, cells were infected with VVT7 and transfected with both the plasmids encoding L (pCR-L) and NP (pCR-NP). The cell lysates were immunoprecipitated with anti-L MAbs (mixture L70-6 and L60-2) or anti-NP MAbs (mixture of 20A and 159-1), and then the immunoprecipitates were analyzed by the Western blot assay. As shown in Fig. 3A(a), the NP–L complex was detected in the immunoprecipitates obtained with either anti-NP or anti-L antibodies [Fig. 3A(a), lanes 1 and 2]. To test whether such complex could be detected in a mixture of the lysates of cells separately transfected with NP or L plasmid, such mixtures were immunoprecipitated with anti-NP MAbs or anti-L MAbs. As shown in Fig. 3A(b) (lanes 5 and 6), the NP–L complex is formed. Furthermore, we tested whether such complex could be formed in a mixture of the L protein expressed in HeLa-CD4$^+$ cells and purified NP protein expressed in *E. coli.* The mixture was immunoprecipitated with anti-NP MAbs or anti-L MAbs. Either anti-NP or anti-L MAbs immunoprecipitated both the NP and L proteins as a complex [Fig. 3A(c), lanes 5 and 6].

To identify domains on the NP protein required for binding to the L protein, we used plasmids encoding the carboxy-terminally truncated NP proteins [Fig. 3B(a)]. Each NP protein or the L protein was synthesized separately in HeLa-CD4$^+$ cells and the cell extracts were then mixed. Subsequently, we tested their ability to form complexes with the L protein by coimmunoprecipitation assays with anti-NP MAb, 20A, or anti-L MAb, L70-6 [Fig. 3B(b) and (c)]. As shown in Fig. 3B(b), complex $NP_{\Delta C50}$–L was detected in the immunoprecipitates obtained with either anti-NP or anti-L antibody [Fig. 3B(b), lanes 5 and 6], but complex $NP_{\Delta C143}$–L cannot be detected [Fig. 3B(c), lanes 3 and 4], indicating that the region of amino acids 403–494 of the NP protein is critical for interaction with the L protein.

DISCUSSION

The L–P complex formation has been described for other negative-strand RNA viruses, including Sendai virus (Horikami *et al.,* 1992; Smallwood *et al.,* 1994), measles virus (Horikami *et al.,* 1994), SV5 (Parks, 1994), VSV (Canter and Perrault, 1996), and rabies virus (Chenik *et al.,* 1998). However, recent reports suggest an important difference in the properties of the P–L polymerase complex of paramyxoviruses and rabdoviruses. Coexpression of the P and L proteins in the same cell was necessary for the L–P complex formation in Sendai virus (Horikami *et al.,* 1992; Smallwood *et al.,* 1994) and measles virus (Horikami *et al.,* 1994), but not for that in VSV (Canter and Perrault, 1996) and rabies virus (Chenik *et al.,* 1998). Sendai virus and measles virus L proteins are unstable unless they are coexpressed with the P proteins (Horikami *et al.,* 1994; Smallwood *et al.,* 1994). On the other hand, the VSV and rabies virus L proteins can be stably expressed in the absence of the P proteins (Horikami *et al.,* 1992, 1994; Smallwood *et al.,* 1994; Canter and Perrault, 1996; Chenik *et al.,* 1998). In the present study, we have shown that the P and L proteins of hPIV-2 could interact with each other in the absence of other virus proteins *in vivo* and *in vitro* by using MAbs specific for the L protein.

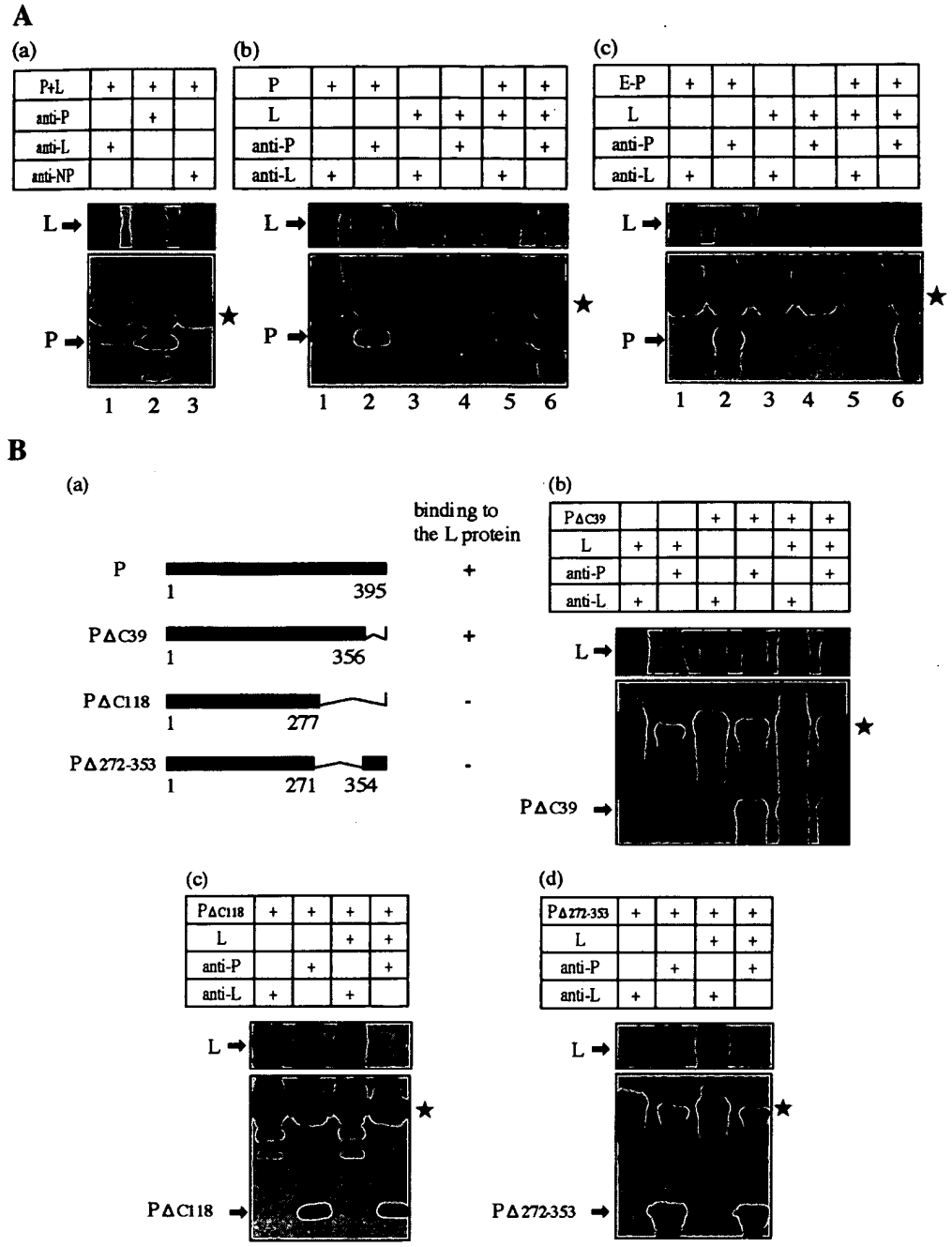

FIG. 2. Analysis of interactions between the L and P proteins by Western blot assay. (A) (a) WT7-infected HeLa-CD4$^+$ cells were cotransfected with the plasmids encoding the full-length P and L proteins. The cell lysates were immunoprecipitated with anti-L MAbs (mixture of L70-6 and L60-2) (lane 1), anti-P MAbs (mixture of 85A and 335A) (lane 2), or anti-NP MAbs (mixture of 20A and 159-1) (lane 3). (b) WT7-infected HeLa-CD4$^+$ cells were transfected with the plasmid encoding the P or L protein. The extracts obtained from the cells transfected with the P (lanes 1 and 2) or L (lanes 3 and 4) plasmid and a mixture of these cell extracts (lanes 5 and 6) were immunoprecipitated with anti-L (lanes 1, 3, and 5) or anti-P MAbs (lanes 2, 4, and 6). (c) WT7-infected HeLa-CD4$^+$ cells were transfected with the plasmid encoding the protein. The bacterially expressed P protein (E-P) (1 μg) (lanes 1 and 2), the extracts of cells transfected with the L plasmid (lanes 3 and 4), and a mixture of the E-P (1 μg) and the extract (lanes 5 and 6) were immunoprecipitated with anti-L (lanes 1, 3, and 5) or anti-P (lanes 2, 4, and 6) MAbs. (B) (a) Schematic representation of the deletion mutants of the P protein and summary of the binding data. Thick lines represent the protein products of each deleted P gene with amino acid positions indicated. Angled lines indicate deleted regions. The construction of plasmids encoding the deleted P proteins is described under Materials and Methods. (b) WT7-infected HeLa-CD4$^+$ cells were transfected with the plasmid encoding the L or $P_{\Delta C39}$ protein. The extracts of cells transfected with the L (lanes 1 and 2) or $P_{\Delta C39}$ (lanes 3 and 4) plasmid, and a mixture of extracts obtained from cells separately transfected with the L and $P_{\Delta C39}$ plasmids (lanes 5 and 6) were immunoprecipitated with anti-L (lanes 1, 3, and 5) or anti-P (lanes 2, 4, and 6) MAbs. (c) WT7-infected HeLa-CD4$^+$ cells were transfected with the plasmid encoding the L or $P_{\Delta C118}$ protein. The extracts of cells transfected with $P_{\Delta C118}$ plasmid (lanes 1 and 2) and a mixture of the extracts of cells separately transfected with the L and $P_{\Delta C118}$ plasmids (lanes 3 and 4) were immunoprecipitated with anti-L (lanes 1 and 3) or anti-P (lanes 2 and 4) MAbs. (d) WT7-infected HeLa-CD4$^+$ cells were transfected with the plasmid encoding the L or $P_{\Delta 272-353}$ protein. The extracts of cells transfected with $P_{\Delta 272-353}$ plasmid (lanes 1 and 2) and a mixture of the extracts of cells separately

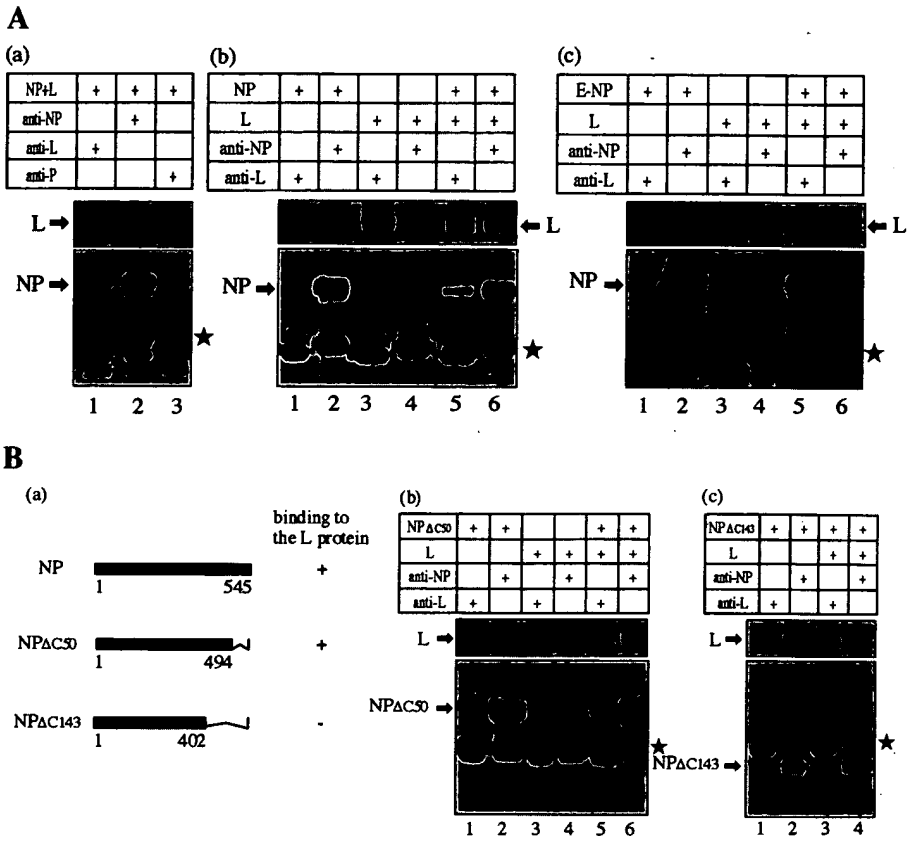

FIG. 3. Analysis of interactions between the L and NP proteins by Western blot assay. (A) (a) VVT7-infected HeLa-CD4$^+$ cells were cotransfected with plasmids encoding the full-length NP and L proteins. The cell lysates were immunoprecipitated with anti-L MAbs (mixture of L70-6 and L60-2) (lane 1), anti-NP MAbs (mixture of 20A and 159-1) (lane 2), or anti-P MAbs (mixture of 85A and 335A) (lane 3). (b) VVT7-infected HeLa-CD4$^+$ cells were transfected with the plasmid encoding the NP or L protein. The extracts obtained from cells transfected with the NP (lanes 1 and 2) or L (lanes 3 and 4) plasmid, and a mixture of the extracts of cells separately transfected with the NP and L plasmids (lanes 5 and 6) were immunoprecipitated with anti-L (lanes 1, 3, and 5) or anti-NP (lanes 2, 4, and 6) MAbs. (c) VVT7-infected HeLa-CD4$^+$ cells were transfected with the plasmid encoding the L protein. At 2 days after transfection, the cell extracts were prepared. The bacterially expressed NP protein (E-NP) (1 μg) (lanes 1 and 2), the extracts of cells transfected with the L plasmid (lanes 3 and 4), and a mixture of the E-NP (1 μg) and the extract (lanes 5 and 6) were immunoprecipitated with anti-L (lanes 1, 3, and 5) or anti-NP (lanes 2, 4, and 6) MAbs. (B) (a) Schematic representation of the truncated NP proteins and summary of the binding data. Thick lines represent the protein products of each truncated NP gene with amino acid positions indicated. Angled lines indicate deleted regions. The construction of plasmids encoding the truncated NP proteins is described under Materials and Methods. (b) VVT7-infected HeLa-CD4$^+$ cells were transfected with the plasmid encoding the L or $NP_{\Delta C50}$ protein. The extracts obtained from cells transfected with the $NP_{\Delta C50}$ (lanes 1 and 2) or L (lanes 3 and 4) plasmid, and a mixture of extracts of cells separately transfected with the L and $NP_{\Delta C50}$ plasmids (lanes 5 and 6) were immunoprecipitated with anti-L (lanes 1, 3, and 5) or anti-NP (lanes 2, 4, and 6) MAbs. (c) VVT7-infected HeLa-CD4$^+$ cells were transfected with the plasmid encoding the L or $NP_{\Delta C143}$ protein. The extracts of cells transfected with $NP_{\Delta C143}$ plasmid (lanes 1 and 2) and a mixture of extracts of cells separately transfected with the L and $NP_{\Delta C143}$ plasmids (lanes 3 and 4) were immunoprecipitated with anti-L (lanes 1 and 3) or anti-NP (lanes 2 and 4) MAbs. The precipitates were separated by LDS-PAGE and electroblotted onto PVDF transfer membranes. Subsequently, the membranes were immunostained with MAbs 20A and L70-6. Stars indicate the positions of immunoglobulin heavy chain.

By using deletion mutants, we have identified a domain on the P protein that is essential for P-L complex formation. The data from these experiments and our previous studies (Nishio *et al.*, 1996, 1997, 1999a) are summarized in Fig. 4. The essential domain for binding to the L protein is located in the carboxy-terminal portion of the P protein, that is, between the domain involved in the P-trimerization and the domain essential for binding to the NP protein. A similar situation exists in the Sendai virus P protein (Smallwood *et al.*, 1994; Curran *et al.*, 1995). Accordingly, the hPIV-2 V protein that shares the N-terminal 164 amino acids with the hPIV-2 P protein, but has a unique C-terminus, does not bind to the hPIV-2 L protein (unpublished

transfected with the L and $P_{\Delta 272-353}$ plasmids (lanes 3 and 4) were immunoprecipitated with anti-L (lanes 1 and 3) or anti-P (lanes 2 and 4) MAbs. The precipitates were separated by LDS-PAGE and electroblotted onto PVDF transfer membranes. Subsequently, the membranes were immunostained with MAbs 85A and L70-6. Stars indicate the positions of immunoglobulin heavy chain.

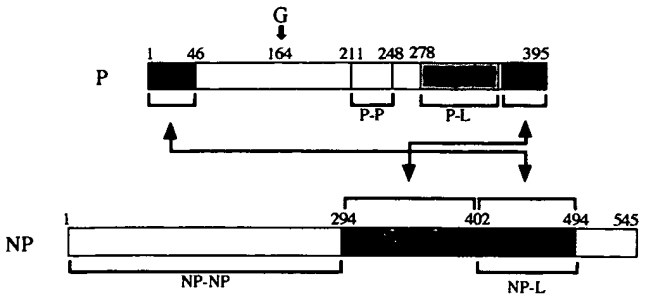

FIG. 4. A schematic model of domains on the P and NP proteins of hPIV-2 that are involved in various protein–protein interactions. The domains identified from this and previous works (Nishio *et al.*, 1996, 1997, 1999a) are indicated. Amino acid (aa) residues 1–46 on the P/V common region are required for binding to the aa 403–494 region of the NP protein. Residues 357–395 on the C-terminal domain of P protein are required for binding to the aa 295–402 region on the NP protein. Residues 211–248 on the P protein are required for its trimerization. Residues 278–353 on the P protein are required for binding to the L protein. N-terminal region (aa 1–294) of the NP protein is required for self-assembly. Residues 403–494 on the NP protein are also required for the binding to the L protein. The P protein is fused in place at residue 164 by an RNA-editing mechanism (insertion of two Gs).

observation). Furthermore, we have demonstrated for the first time that the hPIV-2 protein could form a complex with the NP protein in the absence of other viral proteins *in vivo* and *in vitro*. The region of amino acids 403–494 of the NP protein is essential for the interaction with the L protein. The NP protein synthesized in the bacterial expression system could also form a complex with the L protein. In contrast, the bacterially expressed P protein can form a complex with the NP protein but not with the L protein. These results suggest that phosphorylation of the NP protein is not essential for formation of the NP–L and NP–P complexes, while phosphorylation of the P protein is required for formation of the P–L complex. However, further studies that use the authentic phosphorylated or unphosphorylated P protein will be necessary to establish a definitive conclusion. We have demonstrated for the first time that the L protein interacts directly with the NP protein. However, at the present time, the function of the NP–L complex is unclear. Further experiments will be required to elucidate these relationships and the molecular functions of the complex.

MATERIALS AND METHODS

Plasmid construction

Construction of wild-type L, P, and NP genes. A cDNA clone of the hPIV-2 L, P, or NP gene was inserted into the plasmid expression vector pCR-XL-TOPO (Invitrogen, Carlsbad, CA) downstream of the T7 promoter to obtain plasmids pCR-L, pCR-P, or pCR-NP, respectively, or the bacterial expression vector pCAL-n-EK (Stratagene, La Jolla, CA) to obtain plasmids pCAL-P or pCAL-NP, respectively.

Construction of deleted P or NP genes. The P deletion mutants pCR-$P_{\Delta C39}$, pCR-$P_{\Delta 118}$, and pCR-$P_{\Delta 272-357}$ were generated by PCR amplification using the $pP_{\Delta C39}$, $pP_{\Delta C118}$, and $pP_{\Delta 272-357}$, respectively, as templates as described previously (Nishio *et al.*, 1996, 1997). The NP deletion mutants pCR-$NP_{\Delta C50}$ and pCR-$NP_{\Delta C143}$ were also generated by PCR amplification using the $pNP_{\Delta C50}$ and $pNP_{\Delta C143}$, respectively, as templates as described previously (Nishio *et al.*, 1999a).

Construction of the deleted L gene. The mutant gene encoding amino acids 1–374 of the L protein was cloned into the bacterial expression vector pCAL-n-EK to obtain plasmid pCAL-L_{1-374}.

Purification of recombinantly expressed protein

The plasmid pCAL-P, pCAL-NP, or pCAL-L_{1-374}, which was inserted into the bacterial expression vector pCAL-n-EK, was transferred to *Escherichia coli BL 21 (DE3)* and the expression was induced by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The proteins were expressed as fusion proteins with calmodulin-binding peptide (CBP), and purified as described previously (Nishio *et al.*, 1999b). The purified fusion proteins were cleaved with the site-specific protease EK to remove the CBP tag according to the manufacturer's instructions.

Transfection

HeLa-$CD4^+$ cells (six-well tissue-culture dish) were infected with VVT7 for 1 h at 37°C. The cells were then transfected with various plasmids (3 μg each) by using FuGENE 6 transfection reagent (Roche).

Immunoprecipitation and Western blot assay

After 2 days of transfection, cell extracts were prepared with 150 μl lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 0.6% NP40) containing 4 mM phenylmethylsulfonyl fluoride (PMSF). The samples (150 μl) were incubated with MAbs and protein A–Sepharose for 3 h, and the bound proteins were analyzed by 9% LDS (lithium dodecyl sulfate)–PAGE. Electrophoretic transfer of virus polypeptides from gels onto PVDF transfer membranes was carried out as described previously (Nishio *et al.*, 1996). After washing with PBS, a portion of the membranes to which the P, NP, or mutants protein was transferred were immersed in the methanol–PBS (2:8) containing 4-chloro-1-naphthol (0.3%) and hydrogen peroxide (0.009%), and those to which the L protein was transferred were immersed in ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

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DNAVEC Research Inc., Tsukuba, 305-0856, Japan

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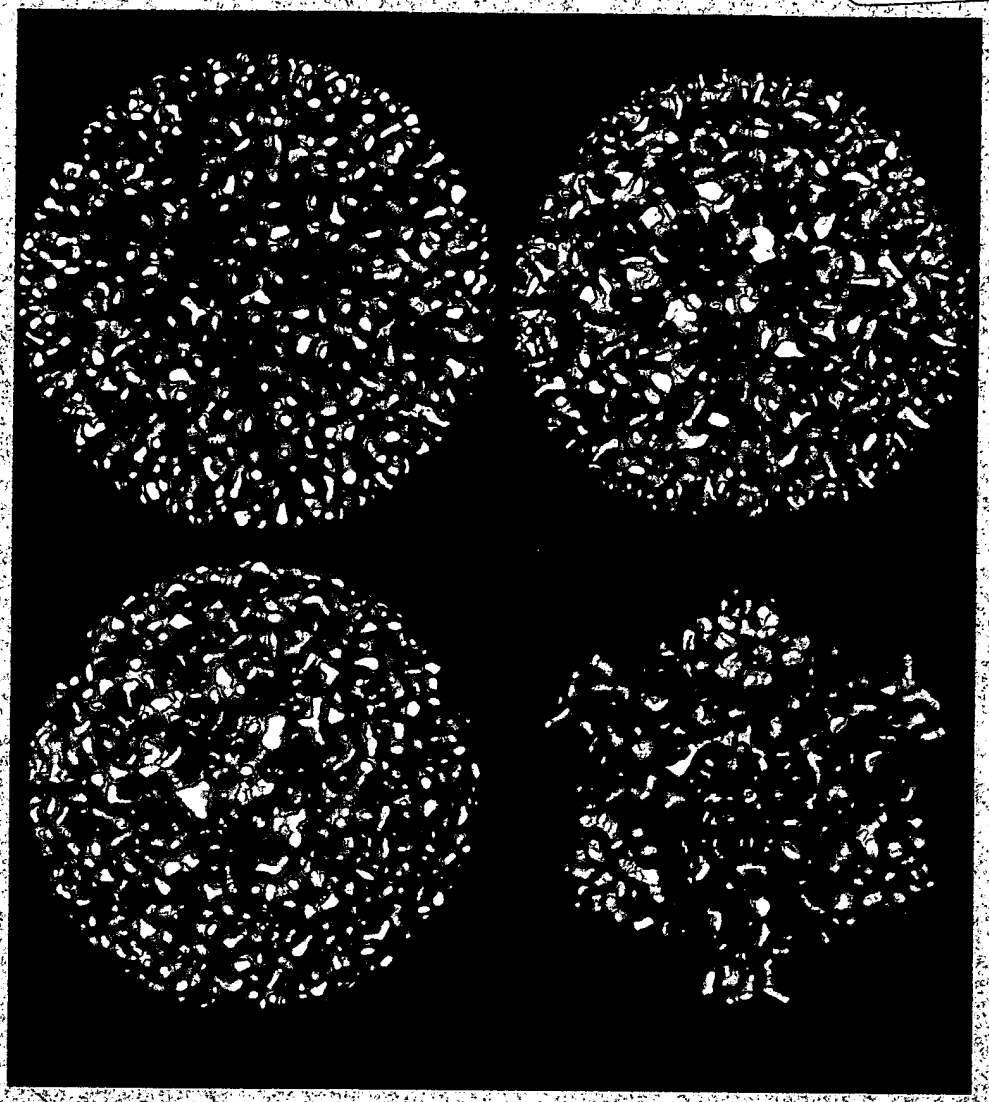
AB We have recovered a virion from defective cDNA of Sendai virus (SeV) that is capable of self-replication but incapable of transmissible-virion prodn. This virion delivers and expresses foreign genes in infected cells, and this is the first report of a gene expression vector derived from a defective viral genome of the **Paramyxoviridae**. First, functional ribonucleoprotein complexes (RNPs) were recovered from SeV cloned cDNA defective in the F (envelope fusion protein) gene, in the presence of plasmids expressing nucleocapsid protein and viral RNA polymerase. Then the RNPs were transfected to the cells inducibly expressing F protein. Virion-like particles thus obtained had a titer of 0.5 .times. 108 to 1.0 .times. 108 cell infectious units/mL and contained F-defective RNA genome. This defective vector amplified specifically in an F-expressing packaging cell line in a trypsin-dependent manner but did not spread to F-nonexpressing cells. This vector infected and expressed an enhanced green fluorescent protein reporter gene in various types of animal and human cells, including nondividing cells, with high efficiency. These results suggest that this ... potential ...



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A Cytoplasmic RNA Vector Derived from Nontransmissible Sendai Virus with Efficient Gene Transfer and Expression

HAI-OU LI,[1] YA-FENG ZHU,[1] MAKOTO ASAKAWA,[1] HIDEKAZU KUMA,[1] TAKAHIRO HIRATA,[1] YASUJI UEDA,[1] YUN-SIK LEE,[1] MASAYUKI FUKUMURA,[1] AKIHIRO IIDA,[1]* ATSUSHI KATO,[2]† YOSHIYUKI NAGAI,[2]‡ AND MAMORU HASEGAWA[1]

DNAVEC Research Inc., Tsukuba-shi, Ibaraki 305-0856,[1] and Department of Viral Infection, Institute of Medical Sciences, University of Tokyo, Minato-ku, Tokyo 108-8639,[2] Japan

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We have recovered a virion from defective cDNA of *Sendai virus* (SeV) that is capable of self-replication but incapable of transmissible-virion production. This virion delivers and expresses foreign genes in infected cells, and this is the first report of a gene expression vector derived from a defective viral genome of the *Paramyxoviridae*. First, functional ribonucleoprotein complexes (RNPs) were recovered from SeV cloned cDNA defective in the F (envelope fusion protein) gene, in the presence of plasmids expressing nucleocapsid protein and viral RNA polymerase. Then the RNPs were transfected to the cells inducibly expressing F protein. Virion-like particles thus obtained had a titer of 0.5×10^8 to 1.0×10^8 cell infectious units/ml and contained F-defective RNA genome. This defective vector amplified specifically in an F-expressing packaging cell line in a trypsin-dependent manner but did not spread to F-nonexpressing cells. This vector infected and expressed an enhanced green fluorescent protein reporter gene in various types of animal and human cells, including nondividing cells, with high efficiency. These results suggest that this vector has great potential for use in human gene therapy and vaccine delivery systems.

Sendai virus (SeV) is an enveloped virus with a nonsegmented negative-strand RNA genome of 15,384 nucleotides and is a member of the family *Paramyxoviridae*. The SeV genome contains six major genes, which are lined up in tandem on a single negative-strand RNA. Three virus-derived proteins, the nucleoprotein (NP), phosphoprotein (P), and large protein (L; the catalytic subunit of the polymerase) form a ribonucleoprotein complex (RNP) with the SeV genomic RNA, and the RNP acts as a template for transcription and replication. Matrix protein (M) engages in the assembly of viral particles. Two envelope glycoproteins, hemagglutinin-neuraminidase (HN) and fusion protein (F), mediate the attachment of virions and penetration of RNPs into infected cells. F protein is synthesized as an inactive precursor protein F_0 and split into F_1 and F_2 by proteolytic cleavage of a trypsin-like enzyme. SeV replication is independent of nuclear functions and does not have a DNA phase. Therefore, it does not transform cells by integrating its genetic information into the cellular genome (16).

Methods to rescue infectious viruses entirely from cloned cDNA have been established for segmented and nonsegmented negative-strand RNA viruses (6, 22, 23, 26). Such reverse genetics technology has enabled the construction of genetically engineered viruses which carry additional foreign genes and opened the way for the development of gene transfer vectors from RNA viruses of this type (24). The vectors prepared by this method have shown a high efficiency of gene transfer and expression of foreign proteins in vitro (3, 12, 18, 21, 28, 32, 36). However, the recombinant paramyxoviruses constructed to date have contained all the viral structural genes and thus are replication competent, giving rise to fully infectious progeny capable of spreading in the body.

Here we report the development of a novel SeV vector that is capable of self-replication but incapable of infecting neighboring cells. The vector does not encode F protein, which is one of the endogenous envelope proteins, but instead incorporates it expressed in *trans*. We further show that an inserted enhanced green fluorescent protein (EGFP) reporter gene is vigorously expressed from this SeV vector in cells of various origins in culture, including human smooth muscle cells, hepatocytes, and lung microvascular endothelial cells, in primary cultures of rat cerebral cortex cells, and in the lateral ventricles and hippocampus of the rat brain. Thus, this F-defective vector appears to represent the important first step toward human gene therapy and vaccine delivery using SeV replicons.

MATERIALS AND METHODS

Virus. The attenuated SeV Z strain was used as a basis for the genome used in this study. Recombinant vaccinia virus vTF7-3 (9) expressing T7 RNA polymerase which had been inactivated with psoralen and long-wave UV light (34) was used for RNP recovery experiments. Recombinant adenovirus AxCANCre (14) expressing Cre recombinase was used for induction of F protein from LLC-MK_2/F7 cells.

Cell culture. A rhesus monkey kidney cell line, LLC-MK_2, was cultured in minimal essential medium (MEM) (Gibco-BRL, Rockville, Md.) supplemented with 10% heat-inactivated fetal calf serum (FCS). For virus propagation, LLC-MK_2/F7 cells were cultured in MEM containing cytosine arabinoside (araC) (Sigma, St. Louis, Mo.) at 40 μg/ml and trypsin (Gibco-BRL) at 7.5 μg/ml. Normal human smooth muscle cells, normal human hepatocyte cells, and normal human lung microvascular endothelial cells (Cell Systems Corp., Kirkland, Wash.) were cultured in SFM CS-C medium (Cell Systems Corp.). All cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.

Plasmid construction. To replace the F gene of SeV cDNA clone with the EGFP reporter gene, the 6.0-kb *Sac*I fragment of pSeV18$^+$b(+) (12) which contained the F gene was cloned into pUC18 (Stratagene, La Jolla, Calif.) to generate pUC18/Sac. A 1,698-bp fragment of the total open reading frame of the F gene in pUC18/Sac was deleted by a combination of PCR and ligation. For an upstream fragment of the F gene, the primer pair FF-1 (5′-GTTGAGTACTG CAAGAGC-3′) and FR-1 (5′-TTTGCCGGCATGCATGTTTCCCAAGGGGA GAGTTTTGCAACC-3′) was used, and for a downstream fragment, the primer pair FF-2 (5′-AAAATGCATGCCGGCAGATGATCACGACCATTATCAGA TGTCTTG-3′) and FR-2 (5′-CTAAAGTACCGCGCGACC-3′) was used (see

* Corresponding author. Mailing address: DNAVEC Research Inc., 1-25-11 Kannondai, Tsukuba-shi, Ibaraki 305-0856, Japan. Phone: 81-298-38-0540. Fax: 81-298-39-1123. E-mail: iida@dnavec.co.jp.

† Present address: Department of Viral Diseases and Vaccine Control, National Institute of Infectious Diseases, Tokyo 208-0011, Japan.

‡ Present address: AIDS Research Center, National Institute of Infectious Diseases, Tokyo 162-8640, Japan.

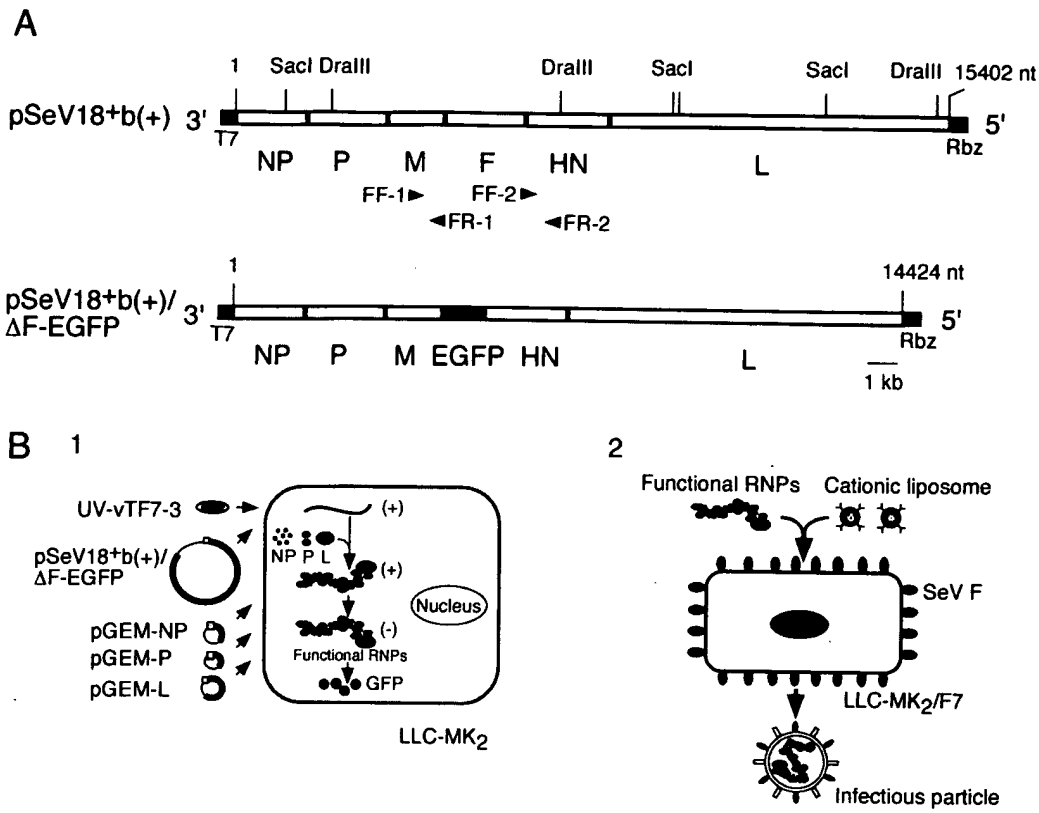

FIG. 1. System for generating the F-defective SeV vector from a cloned SeV cDNA. (A) Schematic representation of the organization of the plasmids pSeV18$^+$b(+), carrying full-length SeV cDNA, and pSeV18$^+$b(+)/ΔF-EGFP, carrying an F-defective SeV cDNA with an EGFP reporter gene. The restriction sites used for construction of pSeV18$^+$b(+)/ΔF-EGFP are indicated. Primers used for PCR amplification are indicated by arrows. T7, T7 promoter; Rbz, hepatitis deltavirus ribozyme sequence; nt, nucleotides. (B) Schematic representation of the two-step procedure for recovery of the F-defective SeV vector. (Panel 1) In the first step, the functional RNPs are recovered in LLC-MK$_2$ cells by using the four plasmids driven by a recombinant vaccinia virus expressing T7 RNA polymerase which had been inactivated with psoralen and long-wave UV light (UV-vTF7-3). (Panel 2) In the second step, RNPs are introduced via a cationic liposome to F-expressing LLC-MK$_2$ cells (LLC-MK$_2$/F7) and produce infectious F-defective virions.

Fig. 1A). The two amplified fragments were digested with *Bsm*I-*Eco*T22I and *Eco*22TI-*Bgl*II, respectively, and ligated with the *Bsm*I-*Bgl*II fragment of pUC18/Sac to generate pUC18/SacΔF. The EGFP gene was amplified by PCR from pEGFP-N1 (Clontech, Palo Alto, Calif.) using a pair of *Nsi*I- or *Ngo*MIV-tagged primers (5′-ATGCATATGGAGATGCGGTTTTGGCAGTAC-3′ [sense] and 5′-TGCCGGCTAATTATTACTTGTACAGCTCGTC-3′ [antisense]). The amplified fragment of EGFP was digested with *Nsi*I and *Ngo*MIV and cloned into the *Nsi*I-*Ngo*MIV sites of pUC18/SacΔF to generate pUC18/SacΔF-EGFP. The 3.4-kb *Dra*III fragment of pUC18/SacΔF-EGFP was replaced with the 4.4-kb *Dra*III fragment of pSeV18$^+$b(+) to generate pSeV18$^+$b(+)/ΔF-EGFP. For the plasmid expressing F protein by the Cre/*loxP*-inducible expression system (1), the 1.8-kb *Sty*I-*Bst*UI fragment of pSeV18$^+$b(+) containing the F gene was blunt ended and inserted into the *Swa*I site of pCALNdLw (1) to generate pCALNdLw/F.

Establishment of F-expressing LLC-MK$_2$/F7 cells. LLC-MK$_2$ cells were transfected with pCALNdLw/F using the mammalian transfection kit (Stratagene) as specified by the manufacturer. G418 (400 μg/ml)-resistant clones were selected after 3 weeks. Expression of F protein was confirmed by infecting the clones with AxCANCre at a multiplicity of infection (MOI) of 3 and analyzed by Western blotting with anti-F monoclonal antibody (MAb) f236 (30) after 3 days. F protein expression on the cell surface was analyzed by flow cytometry after immunostaining with anti-F MAb and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G.

Recovery and amplification of the F-defective SeV vector. Approximately 10^7 LLC-MK$_2$ cells seeded in a 10-cm-diameter dish were infected with psoralen- and long-wave UV-treated vTF7-3 at an MOI of 2. After a 1-h incubation at room temperature, the cells were washed three times with MEM and transfected at room temperature with a plasmid mixture containing pSeV18$^+$b(+)/ΔF-EGFP (12 μg), pGEM-NP (4 μg), pGEM-P (2 μg), and pGEM-L (4 μg) (7) in 110 μl of Superfect transfection reagent (Qiagen, Tokyo, Japan). The transfected cells were maintained for 3 h in 3 ml of OptiMEM (Gibco-BRL) plus 3% FCS, washed three times with MEM, and incubated for 60 h in MEM containing araC (40 μg/ml). GFP expression by the transfected cells was examined by fluorescence microscopy to validate the formation of RNPs inside of the cells. The transfected cells were collected by centrifugation at 1,000 × *g* for 5 min, resuspended in OptiMEM (10^7 cells/ml), and lysed by three cycles of freezing and thawing. Subsequent RNP transfection was performed by mixing the lysate (10^6 cells/100 μl) with 75 μl of OptiMEM and 25 μl of DOSPER (Boehringer Mannheim, Germany) for 15 min at room temperature and then transfecting it into F-expressing LLC-MK$_2$/F7 cells in a 24-well plate. At 24 h after the transfection, the cells were washed three times with MEM and incubated for 3 to 6 days in MEM containing araC (40 μg/ml) and trypsin (7.5 μg/ml). The spread of GFP-expressing cells to neighboring cells was examined by fluorescence microscopy. Virus yield is expressed in PFU and cell infectious units (CIU) (15).

Analysis of viral genomic RNA. Total viral RNA from the F-defective SeV vector or wild-type SeV was isolated using a QIAamp viral RNA mini kit (Qiagen), separated on a 2.2 M formaldehyde–1% agarose gel, transferred to a Hybond N$^+$ membrane (Amersham Pharmacia Biotech, Tokyo, Japan), and hybridized with an F or HN DNA probe generated with a DIG DNA labeling and detection kit (Boehringer). The probes for the F or HN gene were prepared from a 1.8-kb *Sty*I-*Bst*UI or a 1.8-kb *Hha*I-*Dra*I fragment of SeV18$^+$b(+), respectively.

Immunoelectron microscopy. Virus obtained by ultracentrifugation at 10,000 × *g* for 30 min was resuspended in phosphate-buffered saline (PBS) as 10^9 PFU/ml, dropped onto microgrids, dried at room temperature, and fixed with 3.7% formaldehyde for 15 min. Then the grids were treated with anti-F or anti-HN (HN-2) (20) MAb for 60 min, washed three times with PBS, and reacted with gold colloid-labeled anti-mouse immunoglobulin G for 60 min. Treated grids were then washed with PBS, dried, and stained with 4% uranium acetate for 2 min for electron microscopic examination with a JEM-1200EXII instrument (Nippon Denshi, Tokyo, Japan).

Gene transfer to primary cultures of rat cerebral cortex cells. Primary cultures of rat cortical neurons were prepared from E18.5 embryos as described previously (2, 11). Dissociated cells were plated at a density of 80,000 or 100,000/well in eight-well culture slides coated with poly-D-lysine (Becton Dickinson Labware, Bedford, Mass.). The cells were cultured at 37°C in a 5% CO_2 atmosphere for 5 days in neural basal medium enriched with B27 supplement (Gibco-BRL). The F-defective SeV vector was infected at an MOI of 5 and incubated for 3 days. To identify neuronal cells, cells were fixed with 2% paraformaldehyde at room

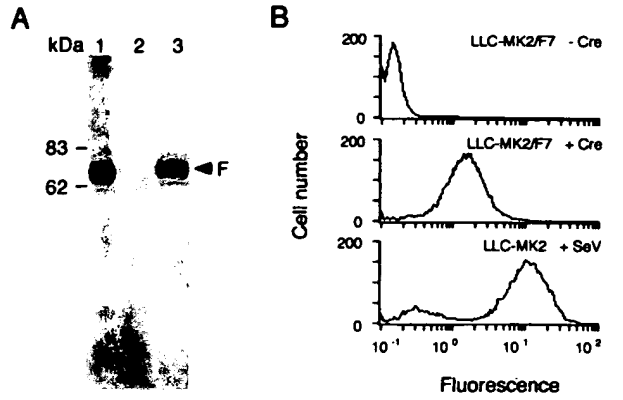

FIG. 2. Inducible expression of F protein in LLC-MK_2/F7 packaging cells. (A) Western blot analysis using anti-SeV F (f-236) MAb. Lanes: 1, LLC-MK_2 infected with wild-type SeV (MOI = 1) for 24 h; 2, LLC-MK_2/F7; 3, LLC-MK_2/F7 infected with adenovirus AxCANCre (MOI = 3) and incubated for 3 days. (B) Flow cytometry analysis of cell surface proteins. Expression of F protein on the packaging cells was examined with the anti-SeV F (f-236) MAb. LLC-MK_2/F7 without induction (top panel), LLC-MK_2/F7 infected with AxCANCre (middle panel), and LLC-MK_2 infected with wild-type SeV (bottom panel) are shown.

temperature for 15 min and immunostained with anti-MAP2 MAb (Boehringer-Mannheim). Immunocytochemistry was performed by indirect-immunofluorescence microscopy (10) with a confocal microscope system (MRC 1024; Nippon Bio-Rad, Tokyo, Japan) using a 470- to 500-nm and 510- to 550-nm excitation band-pass filter on an inverted microscope (Diaphot 30; Nikon, Tokyo, Japan).

Vector injection into rat brain. Female rats, F334/DuCrj (6 weeks old) (Charles River, Ontario, Canada) were anesthetized by intraperitoneal injection of Nembutal (5 mg/kg) and secured on a stereotaxic frame (model 900; David Koph Instruments, Tujunga, Calif.). For intraventricular injection, the burr hole was opened at 5.2 mm off the interaural line toward the bregma and 2.0 mm off lambda toward the right ear. The needle (30 gauge) was inserted 3.6 mm below the surface of the dura. A 20-μl volume of vector suspension (2×10^7 CIU) was injected into the lateral ventricle or hippocampus region.

RESULTS

Construction of F-defective SeV cDNA. F-defective SeV cDNA was constructed by replacing the F gene with an EGFP reporter gene (Fig. 1A). GFP expression was detectable in a single living cell, which allowed us to confirm the successful recovery of RNPs of F-defective SeV inside of such cells.

Construction of a packaging cell line that expresses SeV F protein. SeV F protein is required for the formation of infectious SeV particles. Therefore, recovery of SeV from the RNA genome lacking F gene must be complemented with this gene in *trans*. We therefore constructed an F-expressing packaging LLC-MK_2 cell line with a Cre/*loxP*-inducible expression system. LLC-MK_2 cells were transfected with plasmid pCALNdLw/F, where the F gene is located under the stuffer *neo* sequence flanked by a pair of *loxP* sequences, and stable Neo^r clones were isolated. To these Neo^r clones, a recombinant adenovirus vector, AxCANCre (14), that expresses Cre recombinase was added. Of 15 clones, 7 expressed F protein inducibly; the clone that showed the highest F protein expression (Fig. 2A) was designated LLC-MK_2/F7 and used as a packaging cell line for the F-defective SeV vector. Flow cytometry analysis showed the presence of F protein on the surface of LLC-MK_2/F7 cells (Fig. 2B). The amount of this protein was approximately one-seventh of that on LLC-MK_2 cells infected with wild-type SeV under the same experimental conditions.

Recovery of functional RNPs from an F-defective cDNA. Conventionally, recombinant SeV with the wild-type genome were recovered from cloned cDNAs after infectious particles were rescued in cultured cells and further amplified in embryonated hen eggs or in cultured cells (15). Since infectious particles were not generated from cDNA lacking the F gene in non-F-expressing cells, we have devised a novel rescue procedure which consists of two steps (Fig. 1B). The first step was to recover RNPs of the F-defective RNA genome in LLC-MK_2 cells by using an F-defective cDNA clone and the three plasmids expressing NP, P, and L proteins. GFP-expressing cells were the only RNP-expressing cells on the plate, because such cells were observed only when these four materials were co-transfected into LLC-MK_2 cells. The second step was to transfect RNP into the F-expressing packaging cell line and to collect infectious particles from the supernatants. To raise the efficiency of recovery of RNPs in the first step, we adapted a vaccinia virus vTF7-3 (9) treated with psoralen and long-wave UV irradiation. This treatment inactivated the replication capability of the viruses without impairing their infectivity and T7 RNA polymerase expression. We estimated the recovery frequency by using wild-type SeV cDNA and inoculating the diluted lysates of transfected cells into embryonic hen eggs. With a previous recovery procedure, 1 CIU was detected from 10^5 transfected cells (15). However, with the improved protocol, 1 CIU was detected from only 10^3 cells, indicating an improvement of nearly 100-fold. As for the F-defective SeV cDNA, the numbers of GFP-expressing cells were scored to estimate the efficiency of recovery of functional RNP. Under these conditions, these cells were detected in approximately 1 in 10^5 transfected cells.

FIG. 3. Specific production of the F-defective SeV vector in F-expressing packaging cells in a trypsin-dependent manner. LLC-MK_2 cells (A) or AxCAN Cre-infected LLC-MK_2/F7 cells (B and C) were infected with the F-defective SeV vector and incubated in the presence (A and C) or absence (B) of trypsin. GFP expression by the infected cells was observed by fluorescence microscopy 3 days after infection.

The F-defective SeV vector is specifically propagated in a packaging cell line in a trypsin-dependent manner. The lysates containing functional RNPs were obtained by freeze-thaw cycles, mixed with cationic liposome, and transfected into LLC-MK_2/F7 or LLC-MK_2 cells. The transfected cells were cultured in the presence or absence of trypsin. The infectious virus particles were recovered only from LLC-MK_2/F7 cells cultured with trypsin, suggesting the rescue of infectious virus particles in these cells. The efficiency of recovery at this point was at least 1 CIU from 10^5 transfected cells. In LLC-MK_2/F7 cells cultured in the absence of trypsin or in LLC-MK_2 cells, GFP-expressing cells were detected but did not spread to neighboring cells (Fig. 3). These results showed that the propagation of the F-defective SeV vector and the formation of infectious virus particles are specific to the F-expressing packaging cells and are dependent on trypsin-cleavage. The infectious titer of particles recovered from supernatants of the packaging cells ranged from 0.5×10^8 to 1.0×10^8 CIU/ml.

Confirmation of the genome structure and ultrastructure of the F-defective SeV vector. To examine the genome structure, total RNA from the F-defective SeV vector or wild-type SeV was prepared and analyzed by Northern blot analysis. Probing with the HN gene detected a clear genomic RNA in both F-defective SeV vector and wild-type SeV, but the F-defective SeV vector was smaller than the wild type. When the F gene was used as a probe, no signal was obtained from the F-

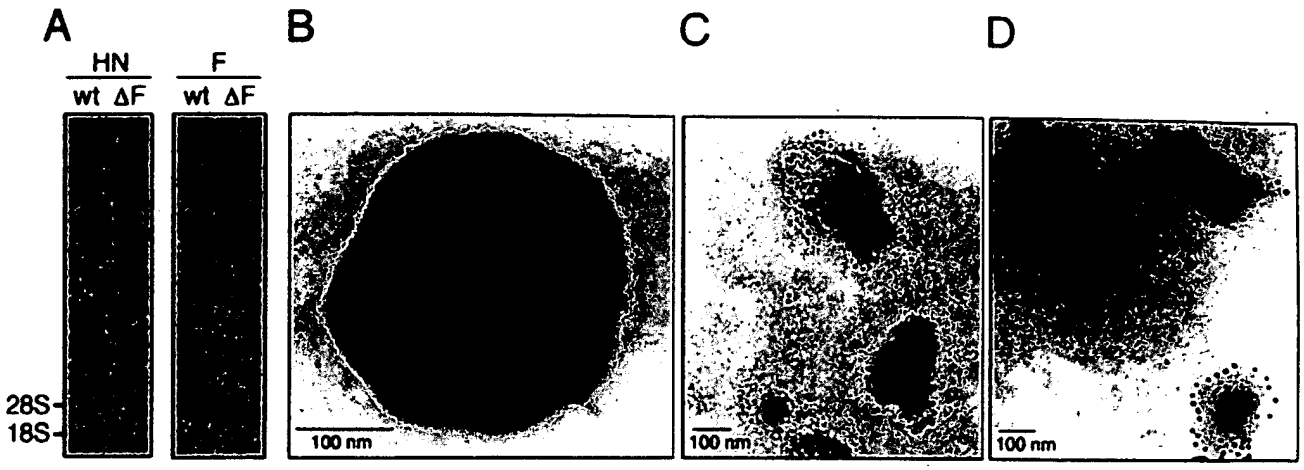

FIG. 4. Structural characterization of the F-defective SeV vector. (A) Northern blot analysis of the RNA genome structure. RNAs from wild-type SeV (wt) and the F-defective SeV vector (ΔF) were prepared and hybridized with cDNA probes of HN (left panel) or F (right panel). The positions of 28S and 18S rRNA are shown. (B to D) Electron microscopic ultrastructure of viral particles. The F-defective SeV vector was negatively stained with phosphotungstic acid (B). The ultrastructure of virus particles after labeling with anti-F (C) or anti-HN (D) MAb and gold-conjugated goat anti-mouse immunoglobulin G is shown.

defective SeV vector but a clear signal was obtained from wild-type SeV (Fig. 4A). The reverse transcription-PCR analysis confirmed the existence of the EGFP gene in the F-deleted region of the F-defective SeV vector (data not shown). These results confirmed that the F-defective SeV vector contains an RNA genome lacking the F gene. Electron microscopic examination of the F-defective SeV vector revealed internally located helical RNP-like structure and an envelope studded with spike-like structures (Fig. 4B). Immunoelectron microscopic examination located the F and HN proteins on the surface of the F-defective SeV vector (Fig. 4C and D).

The F-defective SeV vector efficiently delivers and expresses the EGFP gene in variety of cell types. When primary cultures of neuronal cells derived from fetal rat cerebral cortex were infected with the F-defective SeV vector carrying the EGFP reporter gene at an MOI of 5, nearly 100% of the microtubule-associated protein 2 (MAP2)-positive cells expressed the EGFP reporter gene (Fig. 5A to C). Also, the vector infected and strongly expressed the EGFP gene in almost 100% of normal human hepatocytes, lung microvascular endothelial cells, and smooth muscle cells at an MOI of 3 (Fig. 5D to I). EGFP fluorescence of the infected cells was seen at least from 10 h to 10 days after vector infection. Furthermore, GFP expression was observed in nondividing neuronal cells or ependymal cells of the lateral ventricle when the vector was stereotaxically injected into the hippocampal region or an intraventricular region of rat brain, respectively (Fig. 6). Gene introduction into ependymal cells is of value, since it was reported recently that these cells could be neural stem cells that generate migratory neuronal precursor cells (13). These results showed that the F-defective SeV vector is capable of efficient infection and strong expression of foreign genes in a wide spectrum of cells and tissues.

DISCUSSION

The development of a reverse genetic system has enabled the genetic engineering of negative-strand RNA viruses. This

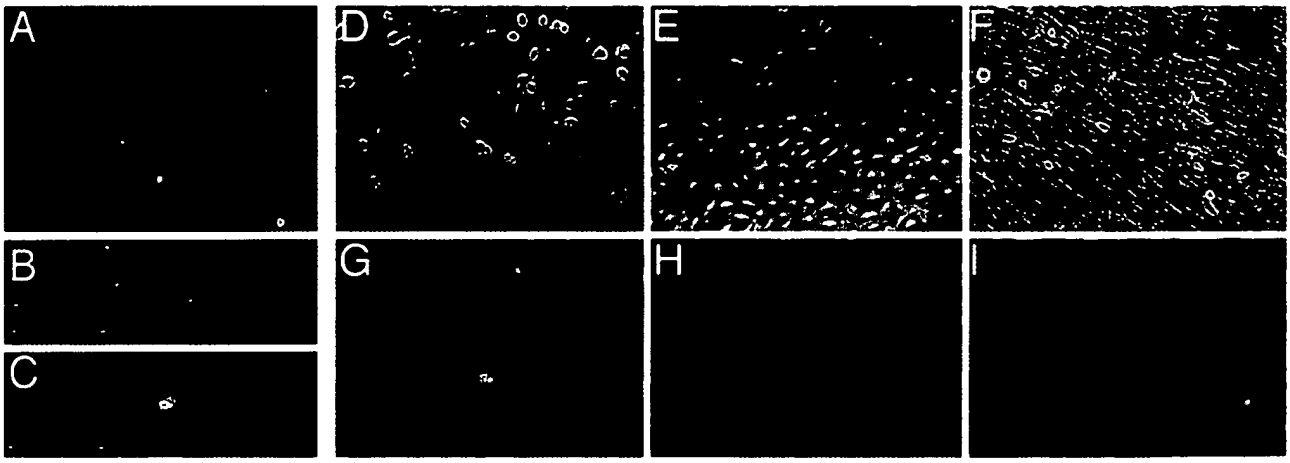

FIG. 5. Introduction and expression of the EGFP gene by the F-defective SeV vector in a variety of cell types in vitro. (A to C) GFP expression by primary neuronal cells derived from rat cerebral cortex 5 days after infection with the vector at an MOI of 5 at lower (A) and higher (C) magnification and immunostained with anti-MAP2 antibody (B). (D to I) Normal human hepatocytes (D and G), normal human lung microvascular endothelial cells (E and H), and normal human smooth muscle cells (F and I) were infected with the F-defective SeV vector at an MOI of 3. GFP expression was observed 3 days after infection (G to I).

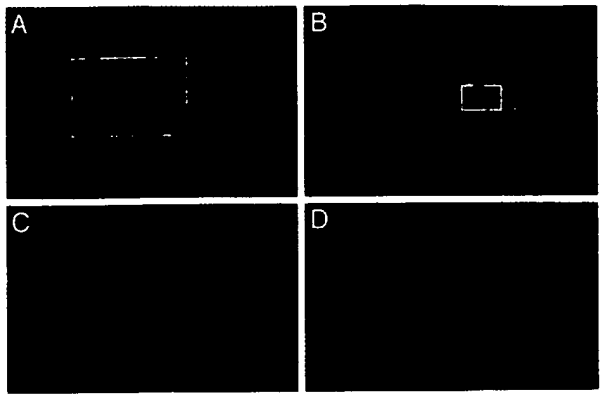

FIG. 6. Gene introduction into the rat central nervous system. The F-defective SeV vector carrying the EGFP gene was injected into rat brain. GFP expression was observed 4 days after vector injection. Fluorescent photomicrographs at lower (A and B) and higher (C and D) magnifications of pyramidal cells of the CA1 region in the hippocampus and ependymal cells of the lateral ventricle.

system has been used to analyze the function of viral genes and to construct recombinant viruses which express foreign proteins. In this study, we made an improvement to this system by devising a new method to generate the F-defective SeV vector from a cloned cDNA of a defective RNA genome. This is the first report on constructing a replicon-based RNA vector in the family *Paramyxoviridae* which replicates in infected cells but does not infect neighboring cells. The improvements achieved in this study are (i) optimization of RNP recovery efficiency by using a UV-inactivated recombinant vaccinia virus expressing T7 RNA polymerase, (ii) construction of an inducible F-expressing packaging LLC-MK_2 cell line supplemented with the F protein in *trans*, and (iii) development of a transfection process for RNP recovered from LLC-MK_2 cells. An attempt to recover the F-defective SeV vector directly in the F-expressing packaging cell line by transfecting F-defective cDNA together with three plasmids expressing NP, P, and L proteins was unsuccessful. Our observation on the gross reduction in F protein expression after vaccinia virus infection of packaging cells suggests that this protein was depleted during this approach (data not shown). The fact that the F-defective SeV vector cannot spread to F-nonexpressing cells indicates that F protein is indispensable for viral infection. Since this system requires the NP, P, and L genes for self-replication and transcription of RNP, a variety of similar self-replicating SeV vectors defective in M, HN, and/or a combination of M, HN, and F genes could be designed if proper complementing cell lines are constructed. Further, we speculate that the strategy developed in this study for rescuing defective viruses is applicable to other negative-strand RNA viruses and represents an innovative method for generation of novel types of vectors.

As to paramyxoviruses carrying defective genome, measles virus defective in M gene were isolated from the brains of subacute sclerosing panencephalitis patients and generated by reverse genetic techniques (4). These viruses were not able to generate progeny viral particles because of the defect in viral envelope assembly but did spread by cell-to-cell fusion. Defective interfering particles of negative-strand RNA viruses which are defective in several viral genes and interfere with the replication of nondefective virus are generated in nature (35). Furthermore, minigenomes in which the entire coding region was replaced with a reporter gene were constructed by genetic engineering in negative-strand RNA viruses (5, 25, 31). Defective interfering particles and minigenomes require helper virus for their replication and virion assembly. The F-defective SeV vector reported in this study is independent of helper virus for its reproduction and is able to self-replicate in infected cells. In the family *Rhabdoviridae*, generation of G-gene-deficient viruses which carry human immunodeficiency virus (HIV) receptor and coreceptor genes has been performed in the vesicular stomatitis virus and rabies virus groups (19, 29). These pseudotyped rhabdoviruses were constructed specifically for targeting to cells infected with HIV-1. Vesicular stomatitis virus has also been used as a vaccine vector (27).

The F-defective SeV vector has several advantages over existing vectors as a gene delivery system for human treatments. (i) SeV is a murine parainfluenza virus, and pathogenicity to humans has not been reported. (ii) This vector replicates exclusively in the cytoplasm of infected cells and does not go through a DNA phase; therefore, there is no concern about unwanted integration of foreign sequences into chromosomal DNA. (iii) This vector has shown a high efficiency of gene transfer and expression of a foreign reporter gene to a wide spectrum of cells and tissues, which is comparable to SeV vectors derived from the wild-type genome. The highest level of expression in mammalian cells has been found in a recombinant SeV expressing HIV-1 envelope glycoprotein gp120 (36). For expression of foreign genes in recombinant F-defective SeV vectors, the genes can be designed as the 3′ proximal first gene of the viruses. A vector with a 3.2-kb foreign gene has been successfully recovered (data not shown). (iv) This vector is not likely to generate wild-type virus in a packaging cell line, since homologous recombination between RNA genomes has not been observed in nonsegmented negative-strand RNA viruses (33). The following studies have confirmed this idea. The F-defective SeV vector was inoculated into embryonated hen eggs or into non-F-expressing LLC-MK_2 cells. The allantoic fluids or the culture supernatants were harvested several days after the vector infection and reinoculated into LLC-MK_2 cells. The presence of infectious viruses in infected cells was examined by GFP expression or immunostaining with an anti-SeV serum. Repeated studies have detected no infectious particles.

Replicon-based vectors derived from positive-strand RNA viruses such as *Sindbis virus* and *Semliki Forest virus* expressed foreign genes with high efficiency, but foreign genes were rapidly lost on passaging of infected supernatant. Also, these vectors had severe cytopathic effects on infected cells (8, 17). The F-defective SeV vector developed in this study is likely to overcome these disadvantages of positive-strand RNA vectors.

One application of this vector is for human gene therapy. The high-level expression of therapeutic genes in wide varieties of cell types, including nondividing types, and the potential safety to humans suggest that this novel vector has great potential for use in transient gene therapy at least (6). Another potential application is in the development of vaccines. This vector resembles DNA vaccines because of its ability to express epitopes of foreign proteins without generating infectious viruses. Therefore, this vector is useful for the design of improved attenuated vaccines. The applications to the treatment of human diseases are now in progress.

ACKNOWLEDGMENTS

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The matrix (M) protein of vesicular stomatitis virus (VSV) was
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expressing the VSV M protein (**SeV**-M-VSV). When cells were
infected with **SeV**-M-VSV, VSV M was found abundantly in the
culture medium. Electron microscopy demonstrated the budding of
two-membraned vesicles (greater than or equal to 0.8 mu m in
diameter) from the infected cells. The outer membrane of the vesicle
was derived from the plasma membran and the inner one possibly
derived from the membrane of an intracellular vesicle. Immune-gold
labeling showed that VSV M was exclusively located in a
double-lay red region. The released membran s were divided into
three parts: the VSV M Vesicles with **SeV** F and HN
[illegible] **SeV** particles, and vesicles associated with
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Double-Layered Membrane Vesicles Released from Mammalian Cells Infected with Sendai Virus Expressing the Matrix Protein of Vesicular Stomatitis Virus

Takemasa Sakaguchi,*,[1] Tsuneo Uchiyama,† Yutaka Fujii,* Katsuhiro Kiyotani,* Atsushi Kato,‡ Yoshiyuki Nagai,‡ Akihiko Kawai,§ and Tetsuya Yoshida*

*Department of Bacteriology, Hiroshima University School of Medicine, Hiroshima 734-8551; †Department of Virology, The University of Tokushima School of Medicine, Tokushima 770-8503; §Department of Pharmaceutical Science, Kyoto University, Kyoto 606-8304; and ‡Department of Viral Infection, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan

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The matrix (M) protein of vesicular stomatitis virus (VSV) was reported to form vesicles on the cell surface and subsequently to be released into the cultured medium when expressed from cDNA by virus vectors. To further investigate VSV M activity, we generated a recombinant Sendai virus (SeV) expressing the VSV M protein (SeV-M^{VSV}). When cells were infected with SeV-M^{VSV}, VSV M was found abundantly in the culture medium. Electron microscopy demonstrated the budding of two-membraned vesicles (≥0.8 μm in diameter) from the infected cells. The outer membrane of the vesicle was derived from the plasma membrane and the inner one possibly derived from the membrane of an intracellular vesicle. Immuno-gold labeling showed that VSV M was exclusively located in a double-layered region. The released membranes were divided into three parts: the VSV M vesicles with SeV F and HN glycoproteins, SeV particles, and vesicles associated with the cytosolic components. The last abundantly contained phosphorylated SeV matrix (M) protein, which is not released in a usual SeV infection. Furthermore the VSV M protein expressed without using a virus vector was efficiently released into the culture medium. These results suggest that the VSV M protein has a budding activity *per se* and that SeV proteins are passively involved in the release of VSV M. © 1999 Academic Press

Key Words: vesicular stomatitis; M protein; Sendai virus; vector; assembly; two-membraned vesicle.

INTRODUCTION

Vesicular stomatitis virus (VSV), a prototype of the Rhabdoviridae, has a bullet-shaped virion enwrapped by a lipid envelope. Inside the virion is a viral nucleocapsid composed of a single-stranded negative-sense RNA genome packed with the nucleocapsid (N) protein, accompanying the minor polymerase components, phosphoprotein (P) and large (L) proteins. VSV has a transmembrane glycoprotein, the G protein, which plays a role in virus attachment to a host cell and in subsequent membrane fusion to initiate infection (Wagner and Rose, 1996). Another major viral protein in the virion is the matrix (M) protein. The M protein has been reported to cause cytopathogenicity during infection. Expression of the VSV M protein from cDNA causes cell rounding by disruption of the cytoskeleton (Blondel *et al.*, 1990; Lyles and McKenzie, 1997). Furthermore the VSV M protein inhibits cellular transcription (Black and Lyles, 1992; Ferran and Lucas Lenard, 1997) and inhibits the nuclear transport of RNAs and proteins by the Ran translocation system (Her *et al.*, 1997). Although the M protein is considered to be multifunctional, the central roles of the M protein are those of virus assembly and morphogenesis. Late in infection, the M protein was shown to interact with the nucleocapsid, inhibiting viral transcription and inducing condensation of the nucleocapsid (Wagner and Rose, 1996). A part of the M protein interacts with the plasma membrane where subsequent virus assembly takes place (Ohno and Ohtake, 1987; McCreedy and Lyles, 1989; Chong and Rose, 1993). The M protein then combines the inner helical nucleocapsid with the envelope (Wagner and Rose, 1996).

In VSV budding, nucleocapsids are incorporated into the skeleton-like structure at the marginal region of the cytoplasm and enwrapped by the plasma membrane and then mature virus particles bud off the cell (Lenard, 1996). The M protein is considered to be involved in all of these viral budding steps. Li *et al.* (1993) showed that when the VSV M protein was expressed without any other VSV protein in insect cells, lipid vesicles containing the VSV M protein were released to the culture medium. It also was shown that the VSV M protein was released as a lipid-associated form when expressed in mammalian cultured cells by using a recombinant vaccinia virus (Justice *et al.*, 1995). In this study, release of mutant M proteins from a temperature-sensitive mutant had a temperature-sensitive phenotype as well, suggesting that

[1] To whom correspondence and reprint requests should be addressed at Department of Bacteriology, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Fax: +81-82-257-5159. E-mail: takemasa@mcai.med.hiroshima-u.ac.jp.

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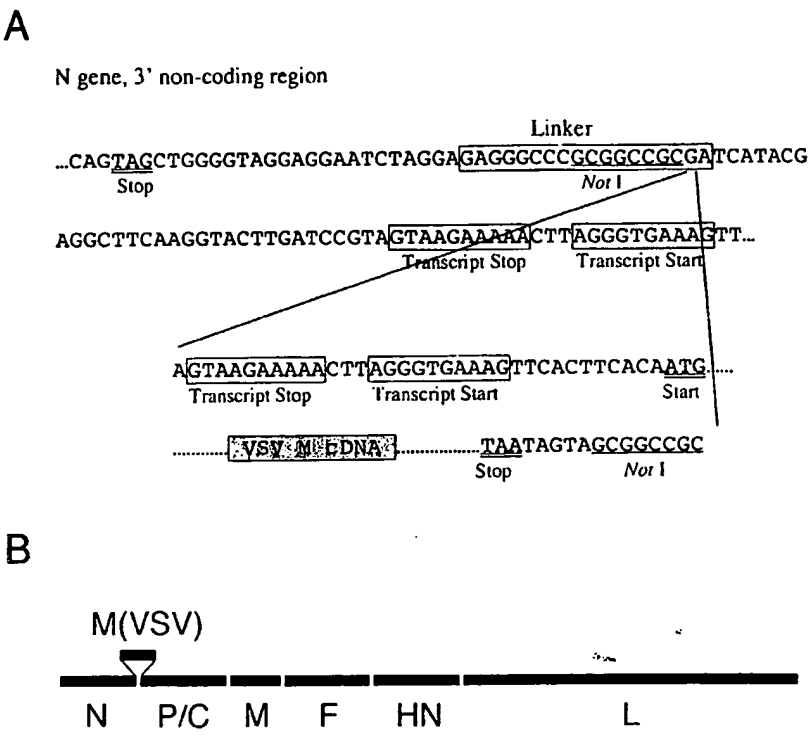


FIG. 1. (A) Nucleotide sequence around the gene insertion site of pSeV18c(+)-M^{VSV}. The VSV M cDNA was inserted into the *NotI* site at the 18 base linker, which was located at the 3' noncoding region of the N gene of SeV genomic DNA. Translation start and stop signals are double underlined, and transcription start and stop signals are boxed. (B) Gene order on pSeV18c(+)-M^{VSV}.

vesicle release by the M protein reflects an actual virus budding in the normal viral life cycle. Hence analysis of the phenomenon may clarify a function of the M protein related to virus budding. In the latter study using mammalian cells, however, electron microscopy encountered difficulty because of the interference by vaccinia virus vectors and liposomes used for transfection (Justice *et al.*, 1995) and detailed observation of the vesicles remained to be elucidated.

Sendai virus (SeV), a prototype of the Paramyxoviridae, is a pleomorphic enveloped virus that has a single-stranded negative-sense RNA as a genome. SeV has two transmembrane glycoproteins, the fusion (F) and hemagglutinin-neuraminidase (HN), in the envelope, and the matrix (M) protein resides between the envelope and the inner nucleocapsid, containing the nucleoprotein (N), phosphoprotein (P), and large (L) proteins (Lamb and Kolakofsky, 1996). SeV has recently been available as a vector to introduce a foreign gene to cells (Hasan *et al.*, 1997), after establishment of the virus recovery system from genomic DNA (Kato *et al.*, 1996). Here we expressed the VSV M protein by a SeV vector to examine the vesicle formation caused by VSV M. We demonstrated by electron microscopy that vesicles are released from the cells expressing VSV M and that the vesicles have double layers of membranes. VSV M was exclusively located in the two-membraned region by immuno-electron microscopy as if two membranes had been combined. We also showed the interaction between the VSV M vesicle and SeV proteins.

RESULTS

Recovery of the SeV carrying the VSV M gene

We generated SeV expressing the VSV M protein. The VSV M gene was inserted between the N and P/C genes of the SeV genomic DNA as an additional gene, accompanying the SeV transcription start and stop signals essential for gene expression [pSeV18c(+)-M^{VSV}]. The nucleotide sequence around the insertion site and gene order of the construct are shown in Fig. 1. A recombinant virus was successfully recovered from the DNA construct and designated as SeV-M^{VSV}.

As reference viruses we used SeV/SpLuc, which had the firefly luciferase gene at a similar position as SeV-M^{VSV} (A. Kato *et al.*, submitted), and SeV wt, which was generated from the wild-type genomic DNA. From another DNA construct, in which the SeV M gene was totally replaced with the VSV M (pSeV(+)-M^{VSV}), no virus was obtained in this recovery system (data not shown), showing no compatibility of the VSV M protein for SeV replication.

The growth efficiency of the recovered virus was investigated by inoculating 1000 cell infectious units (CIU)

of a virus to an embryonated egg and incubating it at 33°C for 3 days. The final infectivity was (7.6 ± 3.9) × 10^7 CIU/ml for SeV-M^{VSV}, (7.7 ± 3.6) × 10^8 CIU/ml for SeV/SpLuc, and (3.0 ± 1.3) × 10^{10} CIU/ml for SeV wt (mean ± standard deviation, four eggs per virus). SeV-M^{VSV} had an approximately one-tenth greater infectivity than SeV/SpLuc, suggesting the harmful effect of the VSV M protein to virus replication. The lower infectivity of SeV/SpLuc compared with SeV wt suggests that insertion of a foreign gene between the N and P/C genes impaired virus growth or it might be due to some inhibitory effect of a luciferase protein to virus replication.

Synthesis of the VSV M protein in SeV-M^{VSV}-infected cells

To examine intracellular synthesis of the VSV M protein by SeV-M^{VSV}, the infected LLC-MK_2 cells were labeled with [^{35}S]Cys and [^{35}S]Met, and the proteins were precipitated with anti-VSV M serum. Analysis of the proteins by SDS–PAGE showed that a specific protein band of 28 kDa was precipitated from the SeV-M^{VSV}-infected cell lysates (Fig. 2A). The protein had the same mobility as the VSV M protein expressed from pCD8-VSV-M, possessing the VSV M cDNA under the T7 promoter, by using the vaccinia virus-mediated T7 transient expression system. This confirmed an expression of the VSV M protein in the SeV-M^{VSV}-infected cells. Immunoprecipitation from an equivalent amount of radiolabeled cell lysates with anti-SeV serum demonstrated that the SeV proteins synthesized in SeV-M^{VSV}-infected cells were fewer than those synthesized in SeV wt-infected cells (Fig. 2A).

We next investigated the morphology of the infected cells. Virus-infected cells were stained with anti-SeV rabbit serum and FITC-conjugated anti-rabbit IgG antibody after 24 h of infection (Fig. 2B). SeV-M^{VSV} infection, in contrast with SeV wt, produced apparent cell rounding in accordance with the cell rounding effect of the VSV M protein (Blondel *et al.*, 1990; Lyles and McKenzie, 1997), indirectly confirming VSV M synthesis in the SeV-M^{VSV}-infected cells. Because immunofluorescent staining with anti-VSV M antibody failed to detect strong signals in the SeV-M^{VSV}-infected cells (data not shown), this raised the possibility that the VSV M protein had been released from the cells as described previously (Li *et al.*, 1993; Justice *et al.*, 1995) and that only small portions remained in the cells.

Protein release from the SeV-M^{VSV}-infected cells to the culture medium

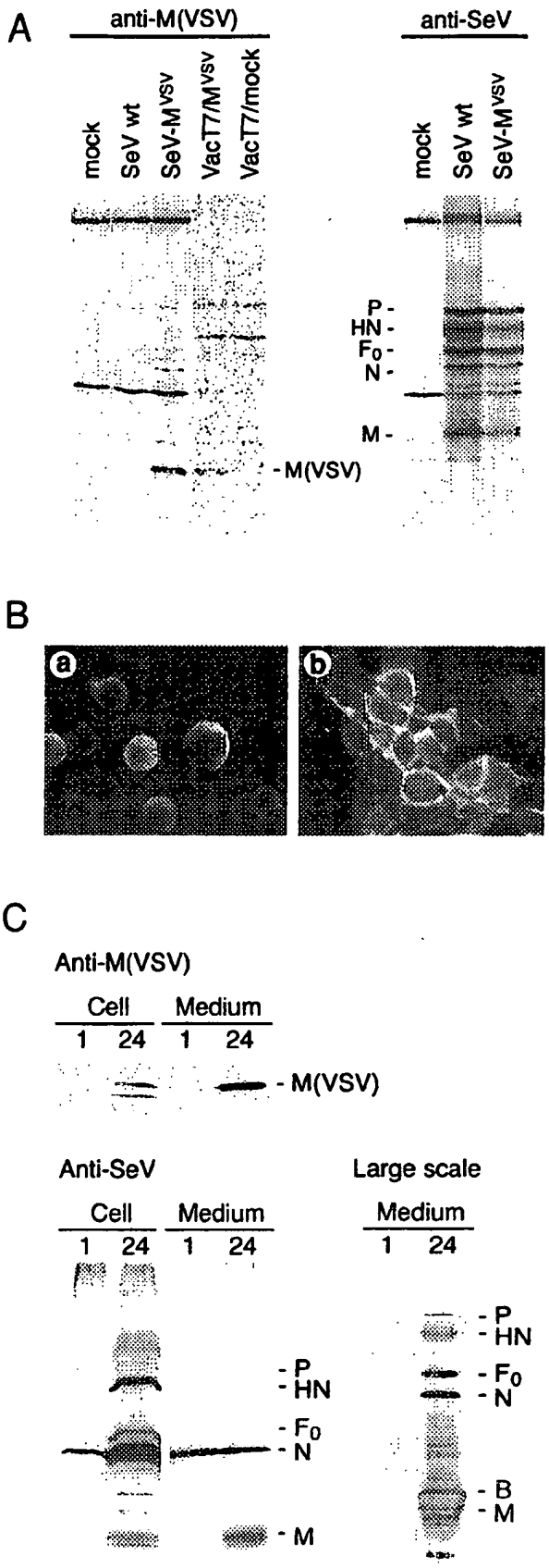
To assess the possibility that the VSV M protein was released to the culture medium, the VSV M protein was quantified by Western blotting. Proteins in the medium or the cell lysates were concentrated, analyzed by SDS–PAGE, and transferred onto a PVDF membrane. Probing the membrane with anti-VSV M serum demonstrated that >75% of the VSV M protein was in the medium and <25% remained in the cells (Fig. 2C). A similar result also was obtained in the BHK-21 cells infected with SeV-M^{VSV} (data not shown). This shows an efficient release of VSV M from cells to the medium. Another possibility that intracellular VSV M is degraded should be noted, however, because some VSV M proteins were partially degraded in the cells (Fig. 2C). When the medium at 24 h p.i. was centrifuged at 100,000*g* for 60 min, the VSV M protein was detected in the pellet, not in the supernatant (data not shown), indicating that the VSV M protein had been released as a precipitable form by ultracentrifugation.

Another set of membranes from the same experiment was probed with anti-SeV serum to analyze the SeV proteins. The SeV M protein was predominant in the medium, whereas all the SeV proteins were detected in the cell (Fig. 2C). To detect the other SeV proteins in the medium, the experimental scale was expanded; the medium from four 10 cm-dishes of the SeV-M^{VSV}-infected cells was concentrated and analyzed in a similar way. The results showed that the other SeV proteins were in the medium though in lower amounts compared with the M protein. This unproportional release of SeV proteins was particular in this system because all of the SeV proteins were proportionally released from the SeV wt-infected LLC-MK_2 cells to the culture medium (data not shown).

Furthermore two species of the SeV M protein could be detected in the medium. It has been shown that the slower migrating band, designated as the B band, represents the M protein phosphorylated at the 70th residue from the N terminus, whereas the faster migrating band represents the unphosphorylated M (Lamb and Choppin, 1977; Sakaguchi *et al.*, 1997). [^{32}P]phosphate labeling of the SeV-M^{VSV}-infected cells demonstrated that phosphorylated M protein was actually released to the medium (data not shown). These results, therefore, indicate that the SeV M protein, including its phosphorylated species, was efficiently released to the medium.

A high level of hemagglutinating activity in the SeV-M^{VSV}-infected medium

The virus infectivity and HA of the culture supernatant were investigated. Confluent monolayers of LLC-MK_2 cells were infected with SeV-M^{VSV}, SeV/SpLuc, or SeV wt at an input m.o.i. of 5. The medium was totally replaced with fresh medium every 12 h, and the accumulated virus in the medium during the period was obtained. An infectivity assay showed that infectious virus was released from the SeV-M^{VSV}- and SeV/SpLuc-infected cells less efficiently than from the SeV wt-infected cells (Fig. 3). This is almost consistent with the results of virus growth in eggs.



HA titers, however, showed a distinct pattern. The HA of SeV-M^{VSV} was as high as that of SeV wt during the course of infection and much higher than that of SeV/SpLuc (Fig. 3). HA was higher than expected from the infectivity in the SeV-M^{VSV} infection; there was a dissociation between infectivity and HA. This raised the possibility that the SeV HN protein was released to the culture medium in a form distinct from virus particles, possibly associating with VSV M.

Electron microscopic observation of the SeV-M^{VSV}-infected LLC-MK$_2$ cells

To further characterize the release of the VSV M protein, the LLC-MK$_2$ cells were infected with SeV-M^{VSV} at an m.o.i. of 5, fixed after 20 h, and observed by electron microscopy. Large vesicles, which were probably in the budding process, were observed on the cell surface (Fig. 4A and 4B). The vesicles had a diameter of 800 ± 500 nm (mean ± standard deviation) by measuring 30 vesicles. The actual diameter could be the same or more than the value. Our study further demonstrated the detailed structure of the vesicle: the plasma membrane and an intracellular vesicle membrane appeared to be gluded at the budding site (Figs. 4C, 4D, and 4F; the adhering site is shown by an arrow). Accordingly, the vesicles had two adhering lipid bilayers: the outer membrane was derived from the plasma membrane and the inner one possibly from an intracellular vesicle.

It was observed that the cytosol was sandwiched between the outer and inner membranes (Figs. 4F and 4G). This may mark an intermediate stage during the formation of the vesicle, or the cytosol may be incorporated into the double-membrane vesicle in the end. It

FIG. 2. (A) Synthesis of the VSV M protein in the SeV-M^{VSV}-infected cells. LLC-MK$_2$ cells were infected with SeV-M^{VSV} or wild-type SeV at an m.o.i. of 10 or mock infected and labeled with [^{35}S]Cys and [^{35}S]Met for 30 min at 7 h p.i. Proteins were precipitated with anti-VSV M antiserum or anti-SeV antiserum and analyzed by 15% SDS–PAGE. A specific protein band (~28 kDa) was found in SeV-M^{VSV} infection when precipitated with anti-VSV M antiserum. For reference, the VSV M protein was expressed from cDNA by the transient expression system with the vaccinia virus possessing the T7 RNA polymerase gene. (B) Morphology of the SeV-M^{VSV}-infected cells. LLC-MK$_2$ cells on a coverslip were infected with SeV-M^{VSV} (a) or SeV wt (b) at an m.o.i. of 5 and incubated for 24 h. The cells were fixed, permealized, and treated with anti-SeV antiserum and FITC-labeled anti-rabbit antibody followed by immunofluorescent microscopy. (C) Release of the VSV M protein and the SeV proteins from the infected cells. LLC-MK$_2$ cells in 6-cm dishes were infected with SeV-M^{VSV} at an m.o.i. of 5, and after 1 or 24 h, the medium and cells were harvested separately. Proteins were concentrated by precipitation with trichloroacetic acid, and an equivalent amount of the proteins was loaded onto 15% SDS–PAGE and transferred to a PVDF membrane. The membrane was incubated with anti-VSV M antiserum or anti-SeV antiserum together with peroxidase-conjugated anti-rabbit IgG antibody, followed by staining with DAB and hydrogen peroxide. (Large scale): the medium from four 10-cm dishes was analyzed in a similar manner.

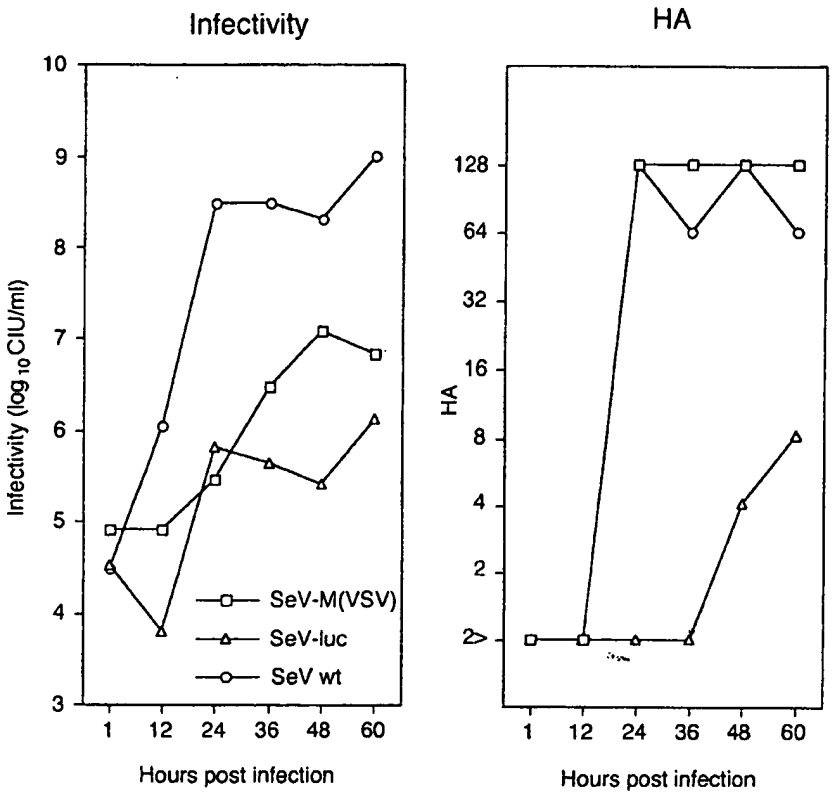


FIG. 3. Dissociation of infectivity and HA of SeV-M^{VSV}. Confluent LLC-MK$_2$ cells in a 3.5-cm dish were infected with SeV-M^{VSV}, SeV/SpLuc, or SeV wt at an m.o.i. of 5 CIU/cell and maintained in 1 ml of MEM. At the time indicated, the medium was replaced with fresh medium, and infectivity and HA in the medium were measured. The means of the two independent experiments were plotted.

also was found that a large vesicle formed a complex shape (Fig. 4H) and that small particles were attached to the vesicle membranes (Figs. 4C, 4F, and 4G). The vesicles seemed to have heterogeneity.

Immunogold-labeling demonstrated that anti-VSV M antisera exclusively reacted with the double-membrane region of the vesicles probably at various steps during budding (Figs. 5A–5C). The antibody did not react with the plasma membrane alone or an intracellular vesicle membrane alone (Fig. 5). It appears that VSV M protein brings together the two layers of the membranes, forming the double-membraned vesicle.

Fractionation of the membranes released from the SeV-M^{VSV}-infected LLC-MK$_2$ cells

We further analyzed the membranes released from the SeV-M^{VSV}-infected cells. The culture medium from the SeV-M^{VSV}-infected cells metabolically labeled with [^{35}S]Cys and [^{35}S]Met was concentrated by ultracentrifugation and then fractionated by sucrose density equibrium ultracentrifugation. Each fraction was processed for immunoprecipitation with a mixture of anti-SeV and anti-VSV M sera and analyzed by SDS–PAGE. The fractions were broadly divided into three portions by distribution of viral proteins (Fig. 6). The fraction of <1.06 g/ml (Fig. 6, fraction 2; top fraction) contained a large amount of VSV M proteins and the SeV F and HN glycoproteins but not SeV M, N, and P proteins. The fractions of 1.11–1.15 g/ml (Fig. 6, fractions 8–10; middle fractions) contained SeV M protein as well as VSV M and SeV glycoproteins but not SeV N and P proteins. The fractions of 1.17–1.19 g/ml (Fig. 6, fractions 14–16; heavy fractions), which had the same density as SeV wt, contained all of the SeV proteins with a trace amount of VSV M protein.

Each of the top, middle, and heavy fractions was pooled and concentrated by ultracentrifugation and observed by electron microscopy. Negative staining showed that the top fraction mainly contained large vesicles (>600 nm in diameter; Figs. 7A and 7B), and thin sectioning demonstrated that the fractions contained highly complex membranous structures (Figs. 7C–7E). These were probably produced when a simple membrane vesicle collapsed and folded during the treatment of samples. Alternatively, this phenomenon may reflect a complex budding form observed in the thin sectioning of the infected cells (Fig. 4H). On the other hand, the heavy fractions contained uniform small particles (<200 nm in diameter; Figs. 8F–8H). Viral spikes and nucleocapsids were observed in the heavy fractions. Considering these findings together with the density and protein profiles

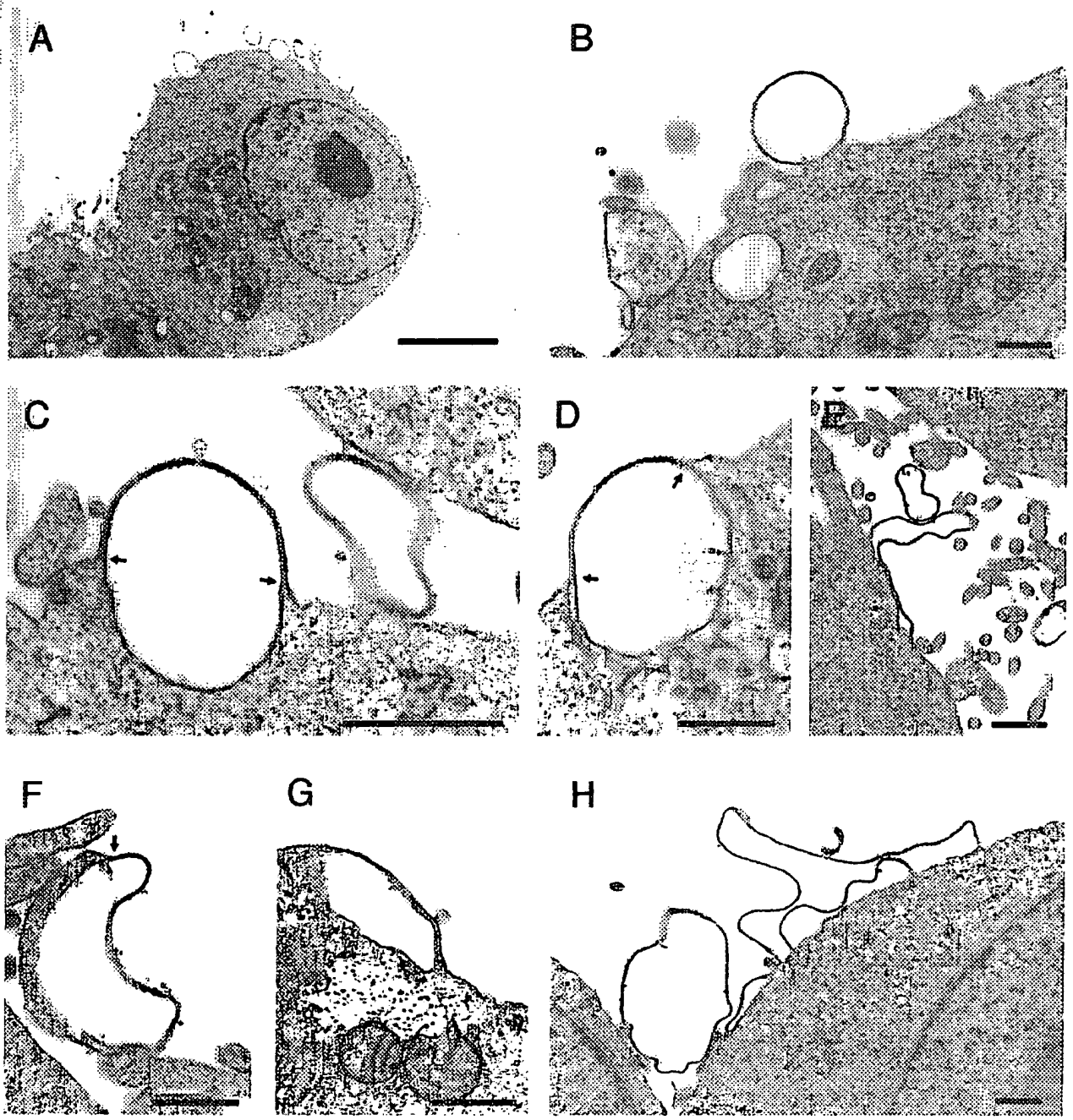


FIG. 4. Vesicles released from the SeV-M^{VSV}-infected cells have two layers of membrane. SeV-M^{VSV} was inoculated to LLC-MK$_2$ cells at an m.o.i. of 5, and after 20 h the cells were fixed with 1% isotonic glutaraldehyde and postfixed with 1% osmium tetroxide. Samples then were dehydrated, embedded in epoxy resin, and sectioned for electron microscopy. The budding vesicles had double membranes, and arrows denote the adhering sites of the two membranes (C, D, and F). It was observed that the cytosol was sandwiched between the outer and inner membranes (F and G); it also was found that a large vesicle formed a complex shape (H), and that small particles attached to the vesicle membranes (C, F, and G). Bars indicate 5 μm in (A) and 500 nm in (B–H).

described above, we concluded that the top fraction contained the vesicles and the heavy fractions contained SeV particles, indicating that vesicles and SeV particles are independently released from the cells. One thing to be noted is that the SeV protein ratio in the heavy fractions is different from that in normal SeV virions. The fractions had less N and P proteins (Fig. 6), suggesting that the trace amount of VSV M interferes with the SeV RNP incorporation to the fractions.

Immunogold labeling in negative staining showed that anti-SeV F or anti-SeV HN monoclonal antibody reacted with the vesicle in the top fraction (Figs. 7A and 7B),

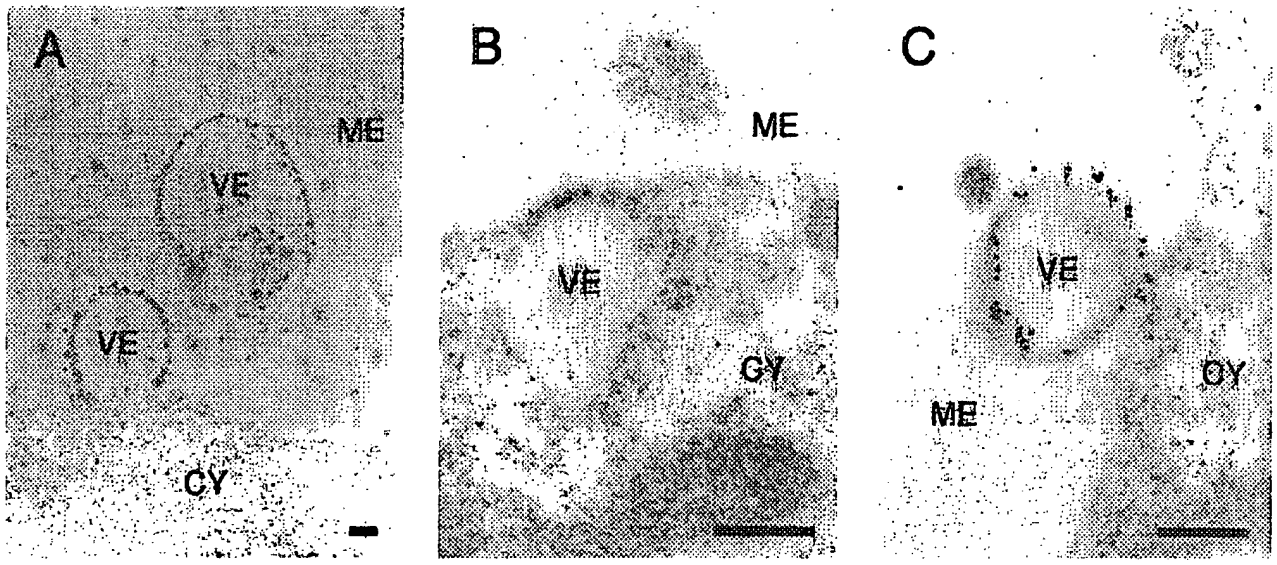


FIG. 5. The VSV M protein resides in the double-membrane region. SeV-M^{VSV} was inoculated to LLC-MK$_2$ cells at an m.o.i. of 5, and the cells were fixed with periodate-lysine-paraformaldehyde fixative after 20 h. The cells then were dehydrated in a graded series of ethanol and embedded in Lowicryl K4M. Ultrathin sections were reacted with anti-VSV M antibody and 10 nm colloidal gold-conjugated anti-rabbit IgG antibody. Anti-VSV M antiserum exclusively reacted with the double membrane region. (A), (B), and (C) may represent various steps of the VSV M budding. ME, medium; VE, vesicle; CY, cytosol. Bar = 200 nm.

suggesting that the vesicle has SeV glycoproteins on its surface. This is consistent with the protein profile of the top fraction (Fig. 6) and a dissociation between infectivity and HA (Fig. 3). The gold particles with a size of 5 nm were clustered, not homogeneous, and the density of the gold particles was not so high as that seen in SeV particles in the heavy fractions (Fig. 8G), showing a heterogeneous and inefficient incorporation of the SeV glycoproteins into the vesicle. Anti-VSV M antibody rarely reacted with the vesicles. This was probably because the VSV-M protein was inside the vesicles or between the two membranes, preventing VSV M from directly interacting with the antibody.

The middle fractions with a density of 1.11–1.15 g/ml contained vesicles more heterogenous in size (200–1000 nm in diameter) (Figs. 8A and 8B). Some vesicles reacted with anti-SeV F or anti-SeV HN but some did not, showing a more heterogenous distribution of SeV glycoproteins (Figs. 8A and 8B). Thin sectioning demonstrated not only membranous structures but also membranes associated with electron dense materials, probably derived from the cytosol (Figs. 8C–8E). This is probably why the middle fractions have a higher density than the top fraction.

The middle fractions were characterized by the presence of a large amount of SeV M protein (Fig. 6). SDS–PAGE in a 10% gel showed that the SeV M protein in the middle fractions migrated more slowly than that in the heavy fraction (Fig. 6, lower panel, band B). This shows that phosphorylated SeV M protein, which was shown to be released to the medium by Western blotting in Fig. 2C, is located in the middle fractions. In a usual SeV infection, only unphosphorylated M protein is incorporated into a virus particle and released to the medium, whereas phosphorylated M remains in the cells (Lamb and Choppin, 1977). This is consistent with the fact that the virions in the heavy fractions mainly have unphosphorylated M protein (Fig. 6, lower panel). Release of the phosphorylated M protein thus is considered to be independent of usual SeV maturation. It should be noted that an additional feature of the middle fractions was the absence of SeV N and P proteins in spite of the presence of SeV M proteins. This suggests that the phosphorylated M protein could not bind to the nucleocapsids.

Autonomous release of VSV M from the expressed cells without using a virus vector

There is a possibility that VSV M is released to the medium with the aid of SeV vector proteins, especially F and HN proteins. To investigate this possibility, we expressed VSV M from plasmid without using a virus vector. The VSV M protein was synthesized in the transfected cells but with lower amounts compared with each of the SeV proteins probably due to its cytotoxic effect. The medium contained a significant amount of VSV M (Fig. 9); ~25% of the synthesized protein was released to the medium. This is compatible with or more than the VSV M released from the SeV-M^{VSV}-infected cells. For the SeV proteins expressed by the same system, significant amounts of F and M proteins were also found in the medium. Considering protein synthesis in the cells, however, only 3.3% of the F protein and 1.8% of the M protein were released into the medium, whereas negligible

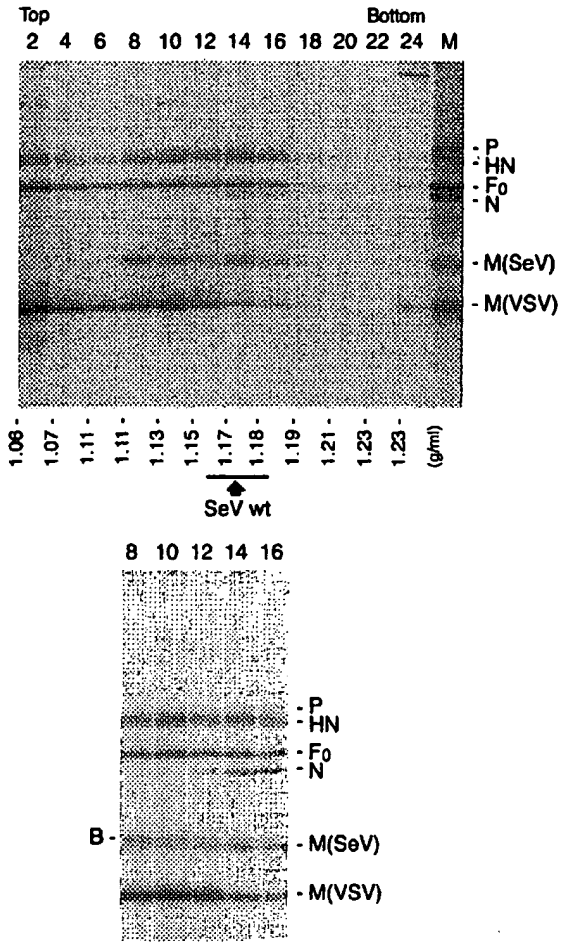


FIG. 6. The released vesicles were broadly divided into three parts by sucrose density gradient ultracentrifugation. Confluent monolayers of LLC-MK_2 cells were infected with SeV-M^{VSV} at an input m.o.i. of 5. At 4 h p.i., the medium was replaced with 5 ml of DMEM containing [^{35}S]Pro-mix and one-fifth concentration of cold cysteine and methionine. At 24 h p.i., infection, the medium was harvested, clarified by a low-speed centrifugation, and processed for ultracentrifugation at 100,000*g* for 60 min. The pellet then was suspended in PBS, and layered on the top of a 20–50% sucrose continuous density gradient, and the tube was centrifuged at 24,000 rpm for 18 h with a SW40 rotor. Proteins in each fraction of even numbers were precipitated with a mixture of anti-SeV and anti-VSV M antisera and analyzed by 15% SDS–PAGE and autoradiography (top). Density of the gradient (g/ml) and the density of wild-type SeV virions are shown below the top panel. The samples from fractions 8–16 were re-analyzed by a 10% gel (bottom). M, immunoprecipitation of the SeV-M^{VSV}-infected cells as a marker.

amounts of the HN, P, or N protein were found in the medium of the expressing cells (Fig. 9). These results show an intrinsic ability of VSV M to go out of the cells. In contrast, the SeV proteins were basically shown to lack a releasing activity, although SeV M and F proteins might have a weak activity. SeV proteins therefore were considered to be released into the culture medium, being implicated in the release of VSV M.

DISCUSSION

In the present study, we expressed the VSV M protein by using a SeV vector. The expressed VSV M was efficiently released to the culture medium. We were able to observe details of the released vesicle by electron microscopy. The vesicles, probably in the budding process, were on the surface of the infected cells. The vesicles had two adhering membranes; one was derived from the plasma membrane and the other possibly from an intracellular vesicle. Immuno-colloidal gold labeling demonstrated that the VSV M protein was exclusively located in the double-membrane region. Thus VSV M appeared to join the two membranes, forming the double-lipid bilayers. The VSV M protein on the inner face of the plasma membrane is considered to bind to intracellular vesicle membranes in the absence of the VSV RNP, and the binding could be a driving force for efficient budding. The release of VSV M after evagination of the plasma membrane has been observed when expressed in insect cells in the absence of other VSV proteins or when a temperature-sensitive mutant of the M protein, tsO23, is propagated at a nonpermissive temperature (Li *et al.*, 1993; Lyles *et al.*, 1996). The vesicles were observed to have thicker membranes than usual and to be "empty," lacking cytoplasmic components such as ribosomes or cytoskeletal elements (Li *et al.*, 1993; Lyles *et al.*, 1996). The present work also explains the membrane thickness and how the cytoplasmic components were excluded from the vesicle.

The origin of the internal layer of the vesicle is unknown. Not so many intracellular large vesicles were found in the cytosol either of the SeV-MVSV-infected cells (Fig. 4A) or uninfected LLC-MK_2 cells (data not shown). The intracellular vesicles seemed to appear under the plasma membrane in the SeV-M^{VSV}-infected cells. One possibility is that the inner layer is derived from the transport vesicles in exocytosis or membrane recycling of endocytosis. In that case, the VSV M protein, which binds to the cytoplasmic surface of the plasma membrane, inhibits fusion of the docked transport vesicles to the plasma membrane. The disturbed transport vesicles might subsequently fuse to each other, forming a large vesicle. This issue should be clarified by the further study.

The present study confirmed and extended previous results about the assembly of VSV and SeV. First, the interaction between the VSV M protein and the SeV glycoproteins were suggested. Sucrose density equilibrium gradient ultracentrifugation separated the released membranes into three parts; the top, middle, and heavy fractions. The top fraction with a low density (1.06 g/ml or less) mainly had large vesicles containing VSV M, SeV F, and HN proteins. SeV glycoproteins were revealed to be incorporated into the vesicles by electron microscopy. A foreign glycoprotein has been shown to be efficiently

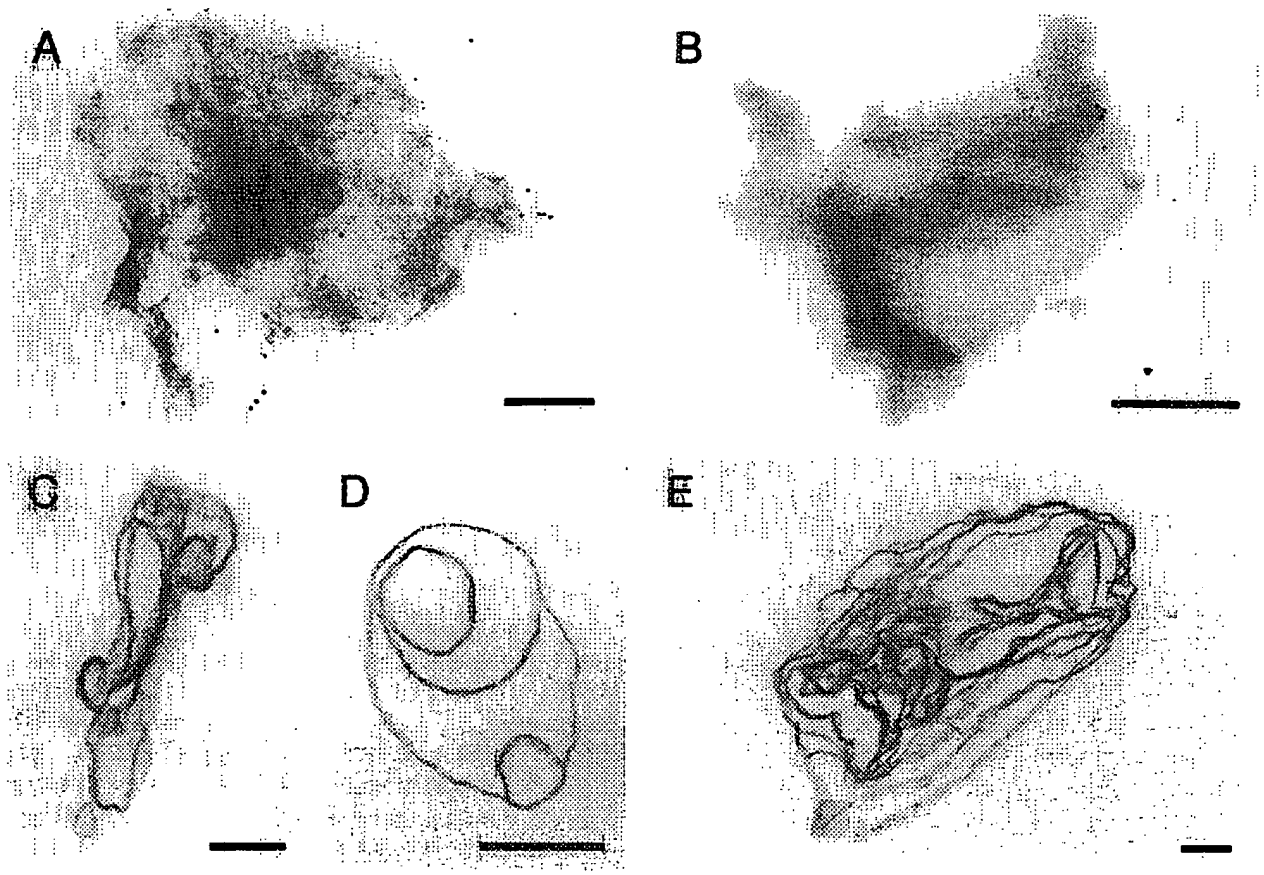


FIG. 7. The top fractions have large membranous structures. The top fractions after sucrose density gradient ultracentrifugation were pooled, diluted with PBS, and recentrifuged at 100,000 *g* for 60 min. The pellet was treated with anti-SeV F (A) or anti-HN mouse monoclonal antibody (B) and 5 nm colloidal gold-conjugated anti-mouse IgG antibody, and simultaneously treated with anti-VSV M antiserum and 10 nm colloidal gold-conjugated anti-rabbit IgG antibody (A and B), followed by negative staining. Alternatively, the pellet was embedded in 1% low-temperature melting agarose and fixed with glutaraldehyde and osmium tetroxide after 24 h. The agarose block was dehydrated, embedded in epoxy resin, and sectioned for electron microscopy (C–E). Bar = 200 nm.

incorporated into the VSV particle, though less efficiently compared with the native G protein (Schnell *et al.*, 1996; Kretzschmar *et al.*, 1997). SeV glycoproteins may be incorporated into the vesicles in a similar manner.

Second, the interaction between the VSV M and SeV M proteins also were suggested, because the middle fractions (1.11–1.15 g/ml) contained a significant amount of the SeV M protein as well as VSV M and SeV F and HN proteins. The interaction between the VSV M and SeV M proteins might be indirect, however, because no evidence was obtained by coprecipitation assay that the two M proteins interact with each other (data not shown). The VSV M protein was efficiently released to the culture medium when expressed without using a viral vector. This denies involvement of the proteins from the viral vectors in the release of VSV M and shows that the VSV M protein has a releasing activity in itself. The SeV proteins in the top and middle fractions are considered to be passively implicated in the release of VSV M.

Third, besides these positive interactions between the VSV M and SeV membrane proteins, negative interactions of VSV M and the phosphorylated SeV M protein with SeV RNP were suggested. Lack of SeV RNP in the top and middle fractions clearly shows the lack of interaction between VSV M and SeV RNP. No SeV RNP was detected in the middle fractions, and the SeV M proteins in the fractions were mainly phosphorylated. We thus suggested that the phosphorylated SeV M cannot bind to the nucleocapsids. This is consistent with the fact that only unphosphorylated M protein is incorporated into a virus particle in a usual SeV infection (Fig. 6; Lamb and Choppin, 1977). On the other hand, the cytosolic components in the middle fractions may be released by associating with the phosphorylated SeV M because it was a particular viral component observed in the middle fractions but not in the other fractions. This is also consistent with an interaction of phosphorylated M with cytoskeletons as described previously (Sanderson *et al.*, 1995).

In conclusion, the VSV M protein was shown to form two-membraned vesicles upon release from the cell. The release of VSV M vesicles was very efficient. Using a SeV expression vector, possible positive or negative interac-

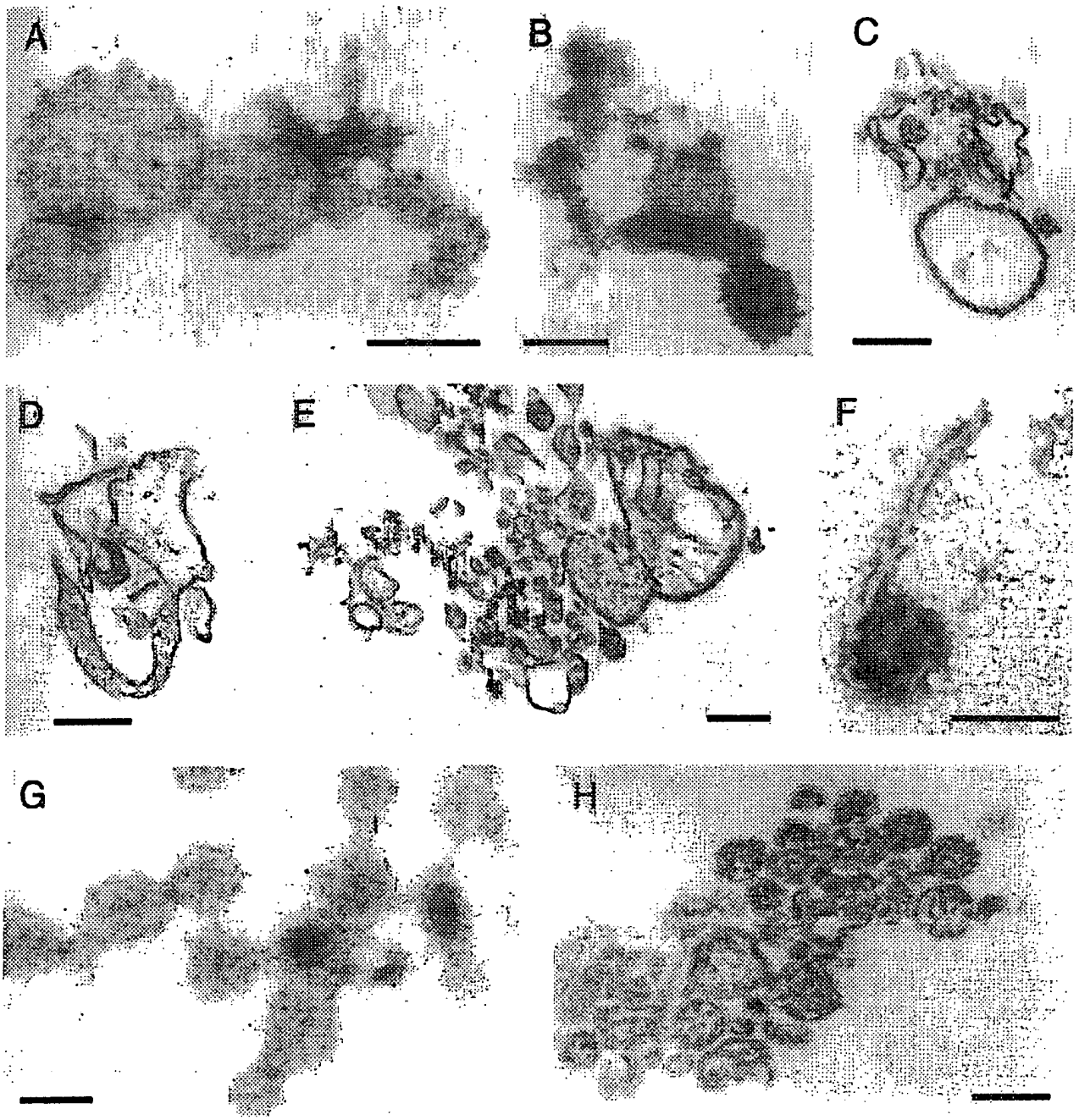


FIG. 8. Electron microscopic observation of the middle and heavy fractions. The middle and heavy fractions after sucrose density gradient ultracentrifugation were pooled, diluted with PBS, and recentrifuged at 100,000 *g* for 60 min. Negative staining and ultrathin sectioning were performed as described in the legend of Fig. 7. Negative staining of the middle fractions after treatment with a mixture of anti-SeV F (A) or anti-HN monoclonal antibodies (B), visualized with 5 nm colloidal gold, and anti-VSV M antiserum, visualized with 10 nm colloidal gold (A and B). Ultrathin sectioning of the middle fractions (C–E). Negative staining of the heavy fractions treated with anti-HN monoclonal antibodies, visualized with 5 nm colloidal, and anti-VSV M antiserum, visualized with 10 nm colloidal gold (F and G). Ultrathin sectioning of the heavy fractions (H). Bar = 200 nm.

tions between VSV M and SeV proteins could be observed, and using a plasmid expression system, VSV M was shown to have a releasing activity *per se*. Investigating the activity of VSV M may lead to an insight into how the protein interacts with host membranes, inducing the formation of double membranes, in further study.

MATERIALS AND METHODS

Cells, viruses, and antibodies

LLC-MK, and BHK-21 cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum. SeV was propagated by inoculation

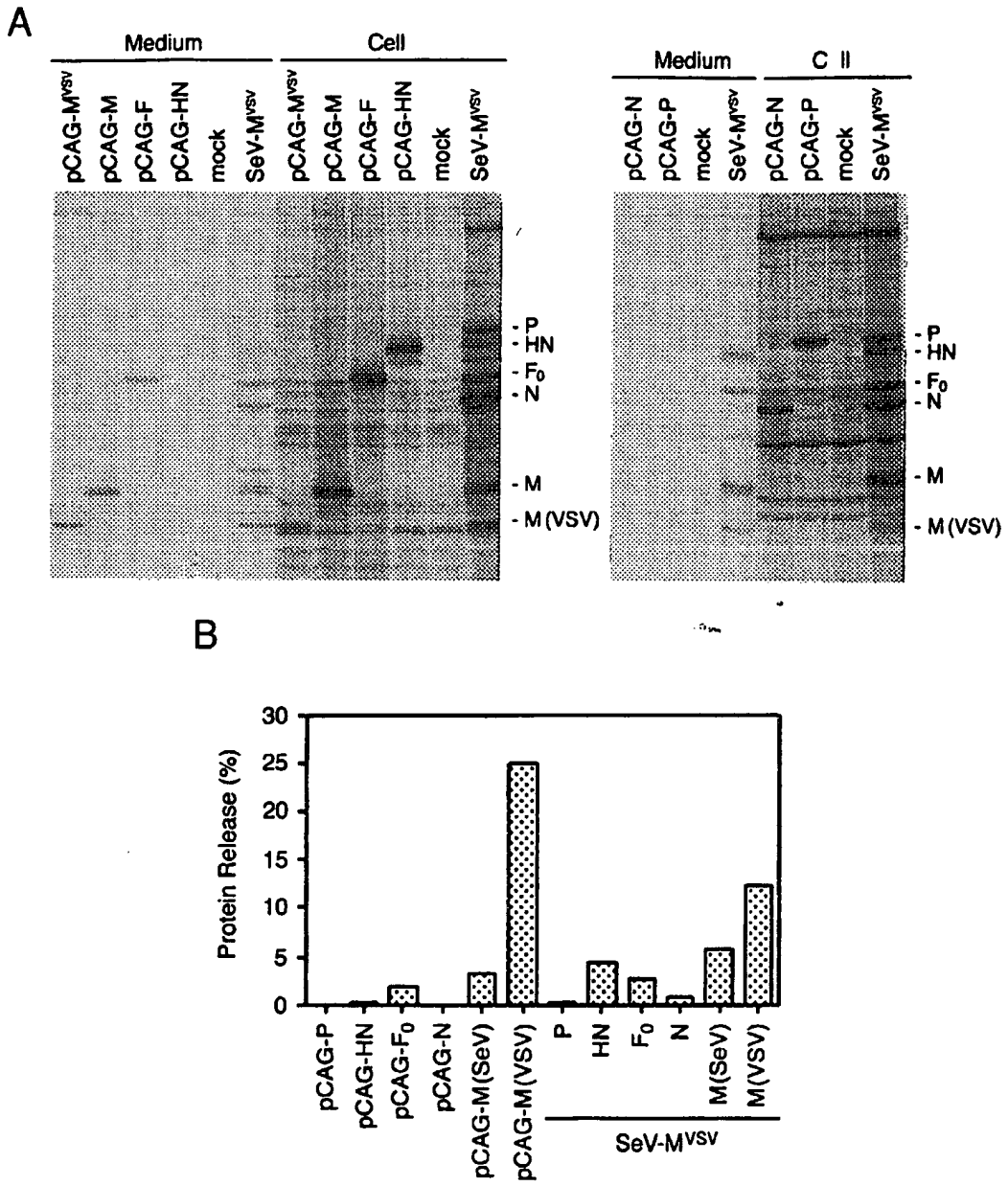


FIG. 9. The VSV M protein is efficiently released from cells when expressed without using a viral vector. (A) The cDNA of the VSV M, SeV M, N, P, F, and HN proteins was subcloned, respectively, into the pCAGGS/MCS plasmid, and LLC-MK_2 cells were transfected with each of the plasmids. At 6 h posttransfection, the medium was replaced with DMEM containing 3.7 MBq/ml of [^{35}S]Pro-mix and $\frac{1}{40}$ diluted cysteine and methionine. At 24 h posttransfection. The medium was mixed with 2× concentrated RIPA buffer, and the cells were lysed with the RIPA buffer, followed by immunoprecipitation as described under Materials and Methods. Proteins were analyzed by 12% SDS–PAGE. SeV-M^{VSV}, SeV-M^{VSV}-infected cells. (B) Proportions of released proteins to the medium. Each value is from two independent experiments.

to 10-day-old embryonated chicken eggs and further incubation at 33°C for 3 days. The infectivity of SeV was measured with an immunofluorescent infectious focus assay (Kiyotani *et al.*, 1990) and expressed as cell infectious units (CIU)/ml. Hemagglutinating activity (HA) was measured by the standard method using a microtiter plate. Recombinant vaccinia virus vTF7.3, carrying the bacteriophage T7 RNA polymerase gene (Fuerst *et al.*, 1986), was provided by Dr. Bernard Moss (National Institute of Health, Bethesda, MD). The vaccinia virus was propagated in CV1 cells, and infectivity was assayed by the standard plaque method. Rabbit antiserum against the VSV M protein was produced by immunizing rabbits with the histidine-tagged VSV M protein expressed in *Escherichia coli*. A mixture of monoclonal antibodies against SeV F protein was prepared from F-49, F-128, and

F-881 and that against SeV HN protein from HN-43, HN-312, and HN-892 (Tozawa *et al.*, 1986).

Plasmids

The cDNA of the M protein of VSV New Jersey serotype was generated by polymerase chain reaction (PCR) following reverse transcription of the genomic RNA, and subcloned to the pCDM8 vector (pCDM8-M^{VSV}). The cDNA was further amplified by PCR using the primers, 5′-ATCTTGCGGCCGCAGTAAGAAAAACTTAGGGTGAAAGTTCACTTCACAATGAGTTCCTTCAAGAA-3′ and 5′-AGTTAGCGGCCGCTACTATTACTTAAATGGACTCAA-3′, and subcloned into the *Not*I site of pSeV18c(+). The plasmid has the whole SeV genome DNA flanked by the T7 promoter and the hepatitis delta ribozyme, and the 18 base *Not*I linker inserted at the 3′ noncoding region of the N gene (Fig. 1A; A. Kato *et al.*, submitted). The resultant plasmid, pSeV18c(+)-M^{VSV}, has the VSV M cDNA between the N and P/C genes (Fig. 1B). The nucleotide sequence of the VSV M gene including the insertion site was confirmed after subcloning. Another DNA construct, in which the SeV M gene was totally replaced with the VSV M cDNA [pSeV(+)-M^{VSV}], was constructed as described previously (Sakaguchi *et al.*, 1997). The VSV M cDNA also was subcloned into a mammalian expression vector, pCAGGS/MCS, which has the cytomegalovirus immediate early enhancer and the chicken β-actin promoter upstream of a multicloning site (Niwa *et al.*, 1991).

Recovery of SeV from cDNA

SeV was recovered from pSeV18c(+)-M^{VSV} as described by Kato *et al.* (1996). Briefly, 1.2×10^7 of LLC-MK_2 cells in a 6-cm dish were infected with vTF7.3 at an m.o.i. of 2 PFU/cell, and transfected with pSeV18c(+)-M^{VSV} (10 μg) and the plasmids encoding *trans*-acting proteins, pGEM-N (5 μg), pGEM-P (2.5 μg), and pGEM-L (5 μg) with the aid of a liposomal transfection reagent, DOTAP (Boehringer-Mannheim, Mannheim, Germany). The cells then were maintained in MEM containing 40 μg/ml of cytosine β-D-arabinofuranoside (Ara-C; Sigma, St. Louis, MO) and 100 μg/ml of rifampicin (Sigma). After 2 days, the cells were suspended in 1 ml of Dulbecco's phosphate-buffered saline (PBS) and disrupted with a brief sonication. The cell lysates were inoculated to 10-day-old embryonated chicken eggs.

Protein analysis by metabolic labeling and immunoprecipitation

Confluent monolayers of LLC-MK_2 cells in a 3.5-cm dish were infected with SeV at an m.o.i. of 20. For protein expression from cDNA, subconfluent monolayers of LLC-MK_2 cells were infected with vaccinia virus vTF7.3 at an m.o.i. of 10 PFU/cell and transfected with plasmid DNA by using DOTAP. After 6 h, the cells were labeled with ^{35}S[Cys] and [^{35}S]Met ([^{35}S]Pro-mix; 3.7 MBq/ml; Amersham, Arlington Heights, IL) for 30 min in methionine- and cysteine-free Dulbecco's modified MEM (DMEM; Life Technologies, Rockville, MD). The cells were lysed in radioimmuno-precipitation assay (RIPA) buffer [10 mM Tris–HCl, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 50 mM iodoacetamide, and 1 mM PMSF (phenylmethanesulfonyl fluoride)]. Polypeptides were immunoprecipitated with anti-SeV serum or anti-VSV M antiserum and analyzed by 15 or 10% SDS–polyacrylamide gel electrophoresis (PAGE) as described previously (Sakaguchi *et al.*, 1996). Radioactivity was analyzed by using a Fujix BAS 2000 image analyzer (Fuji Photo Film, Tokyo, Japan).

For protein expression from plasmid without using a virus vector, the pCAGGS/MCS plasmid was introduced to subconfluent LLC-MK_2 cells in a 6-cm dish with the lipofectAMINE and PLUS reagents (Life Technologies) following the manufacturer's instruction. After 6 h post-transfection, the cells were labeled for 20 h with 3.7 MBq/ml of [^{35}S]Pro-mix in DMEM in which cysteine and methionine concentrations were reduced to $\frac{1}{40}$. The medium was mixed with an equal volume of 2× concentrated RIPA buffer, and the cells were lysed in RIPA buffer. The proteins were further immunoprecipitated and analyzed as described above.

Immunofluorescent staining of the infected cells

LLC-MK_2 cells on a coverslip were infected with SeV at an m.o.i. of 5, and incubated for 24 h in MEM. The cells were fixed with 1% formalin in PBS for 20 min at room temperature, permealized with 0.1% Triton X-100 for 2 min, and sequentially incubated with anti-SeV serum and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (Organon Teknika, West Chester, PA). Then the cells were observed with a VANOX-T fluorescent microscope (Olympus, Tokyo, Japan).

Western blotting

LLC-MK_2 cells in 6-cm dishes were infected with SeV-M^{VSV} at an m.o.i. of 5, and the medium and cells were harvested separately after 1 or 24 h. The medium was clarified by a low-speed centrifugation, and the cells were solubilized with RIPA buffer and clarified by centrifugation at 15,000 rpm for 20 min in a microfuge. The proteins in the fractions were concentrated by precipitation with trichloroacetic acid. An equivalent amount of the precipitated pellet was loaded onto 15% SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was probed with anti-VSV M antiserum and peroxidase-conjugated anti-rabbit IgG antibody (Organon Teknika), followed by visualization with 3,3′-diaminobenzidine (Dojindo, Kumamoto, Japan) and hydrogen peroxide.

Sucrose density gradient equibrium ultracentrifugation

Confluent monolayers of LLC-MK_2 cells in four 10-cm dishes were infected with SeV-M^{VSV} at an input m.o.i. of 5. At 4 h p.i., the medium was replaced with 5 ml of DMEM that had 740 kBq/ml of [^{35}S]Pro-mix and one-fifth concentration of cold cysteine and methionine. At 24 h p.i., the medium was harvested, clarified by a low-speed centrifugation, and ultracentrifuged at 100,000*g* for 60 min. The pellet then was suspended in a small volume of PBS, layered on the top of a 20–50% sucrose linear density gradient, and centrifuged at 24,000 rpm for 18 h in a SW40 rotor (Beckman, Palo Alto, CA). After the spin, fractions were taken with an Auto Densi-Flow fractionator (Labconco, Kansas City, MO). Each fraction (0.5 ml) was mixed with an equal volume of 2× concentrated RIPA buffer, and the proteins were immunoprecipitated with a mixture of anti-SeV and anti-VSV M antisera, and analyzed by 15% SDS–PAGE and autoradiography.

Electron microscopy

Electron microscopy was performed as described previously (Uchiyama and Uchida, 1988) with some modification. The infected cells were washed with PBS and fixed with 1% isotonic glutaraldehyde for 1 h on ice. For observation of sucrose fractions, the fractions were prepared from unlabeled SeV-M^{VSV}-infected cells in 27 of 10-cm dishes as described above. A part of the fractions was analyzed by SDS–PAGE followed by silver staining. Fractions of a similar protein profile were pooled, diluted with PBS, and recentrifuged at 100,000*g* for 60 min. The pellet was resuspended in a small volume of PBS and embedded in a 1% low-melting-temperature agarose, followed by fixation. The fixed cells or the agarose blocks were postfixed with 1% osmium tetroxide for 1 h on ice. The specimens then were dehydrated in a graded series of ethanol, immersed in propylene oxide, and embedded in Epon 812 (TAAB, Aldermaston, England). Ultrathin sections were examined under a JEOL JEM-1200EX II transmission electron microscope.

Immunoelectron microscopy

The sucrose fractionated samples prepared as described above were placed on Formvar carbon-coated nickel grids and reacted with a mixture of mouse monoclonal antibody to HN or F of SeV and rabbit antiserum to VSV M followed by a reaction with a mixture of colloidal gold (5 nm in mean diameter)-labeled antibody to mouse IgG and colloidal gold (10 nm in mean diameter)-conjugated antibody to rabbit IgG (Amersham). The grids then were stained with 4% uranyl acetate.

Immunoelectron microscopy of infected cells was performed basically as described previously (Uchiyama *et al.*, 1994). Briefly, the infected cells were washed with PBS and fixed with periodate-lysine-paraformaldehyde fixative on ice. The fixed cells were dehydrated in a graded series of ethanol and embedded in Lowicryl K4M (Electron Microscopy Sciences, Ft. Washington, PA) at −30°C. Ultrathin sections on grids were reacted with rabbit antiserum against VSV M followed by a reaction with colloidal gold (10 nm in mean diameter)-conjugated antibody to rabbit IgG. The ultrathin sections then were stained with uranyl acetate and observed under a transmission electron microscope.

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virions. The use a **Sendai virus vector** has therefore been shown instrumental in the identification of mutant M proteins interfering with the viral assembly-budding process. (C) 1996 Academic Press, Inc.

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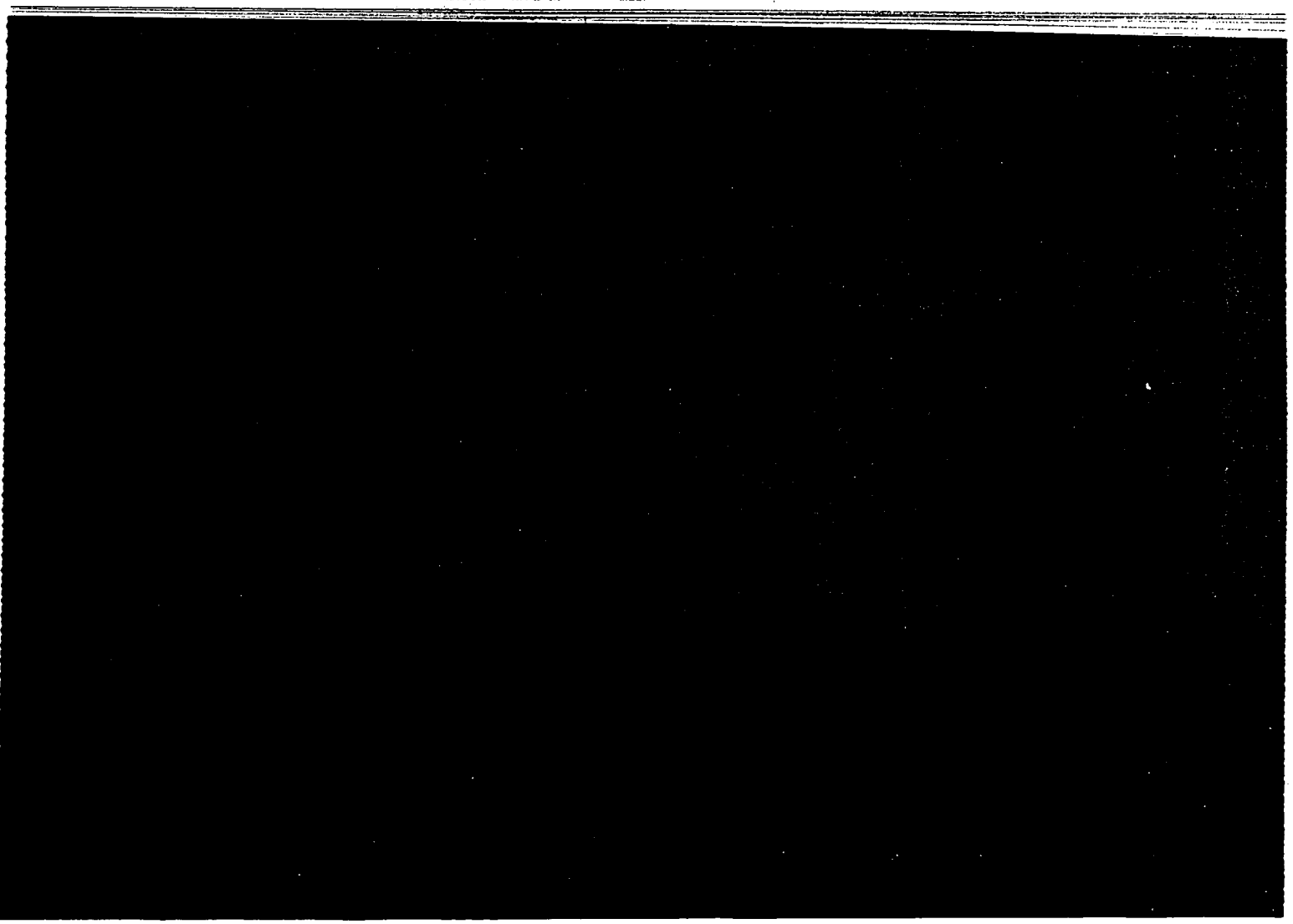

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The nucleoprotein of Marburg virus is phosphorylated

Stephan Becker, Sabine Huppertz, Hans-Dieter Klenk and Heinz Feldmann*†

Institut für Virologie, Robert-Koch-Straße 17, 35037 Marburg, Germany

The nucleoprotein (NP) of Marburg virus (MBG), a filovirus, is encoded by the gene closest to the 3′ end of the non-segmented negative-strand RNA genome. Sequence comparison has indicated that NP is the functional equivalent to the nucleoproteins of paramyxoviruses and rhabdoviruses. Expression of recombinant NP in two eukaryotic systems using vaccinia virus and baculovirus (vectors pSC11 and pAcYMB1, respectively) and analysis of MBG-specific proteins have demonstrated that the NP of MBG is phosphorylated. The NP appeared in two forms differing in M_r by about 2K (94K and 92K respectively). Dephosphorylation clearly demonstrated that the 94K form is phosphorylated whereas the 92K form is unphosphorylated. In virion particles NP was exclusively present in the phosphorylated form. These findings suggest that only the phosphorylated NP can form nucleocapsid complexes and interact with the genomic RNA.

Introduction

The family *Filoviridae* accommodates Marburg virus (MBG), the prototype of the family, Ebola virus (EBO) and Reston virus (RES) (Kiley *et al.*, 1982; Murphy *et al.*, 1990). Filoviruses are classified in the order *Mononegavirales* (Pringle, 1991) which also contains the non-segmented negative-strand (NNS) RNA virus families *Paramyxoviridae* and *Rhabdoviridae*. MBG and EBO are extremely pathogenic for humans and non-human primates, causing a severe haemorrhagic disease with high mortality rates.

MBG particles consist of at least seven structural proteins: the L protein (Mühlberger *et al.*, 1992), the glycoprotein (GP) (Will *et al.*, 1993), the nucleoprotein (NP) (94K) (Sanchez *et al.*, 1992), and four viral structural proteins (VP) with M_rs of 38K (VP40), 32K (VP35), 28K (VP30) and 24K (VP24) (Kiley *et al.*, 1988). The corresponding genes are located on the NNS RNA genome in the following linear order: 3′ untranslated region–NP–VP35–VP40–GP–VP30–VP24–L–5′ untranslated region (Feldmann *et al.*, 1992).

The mature NP in MBG particles has an M_r of 94K and is the major component of the viral nucleocapsid. It represents approximately 27% of the virion protein mass (Kiley *et al.*, 1988). The nucleic acid sequence of the NP gene has been recently elucidated (Sanchez *et al.*, 1992). Comparison of the deduced amino acid sequence showed a high degree of homology to the N-terminal 400 amino acids of the EBO NP. A small region in the middle of the NP sequence was found to contain a significant homology with the nucleoproteins of paramyxoviruses and to a lesser extent with those of rhabdoviruses (Barr *et al.*, 1991; Sanchez *et al.*, 1992).

Little is known about the mode of transcription and replication of filoviruses and their unusually high pathogenicity. Detailed knowledge of the functions of the various MBG proteins, their co- and post-translational modifications, and their interactions with virion or cellular proteins or nucleic acids would create a foundation for further investigations. Studies on paramyxoviruses and rhabdoviruses have shown that the nucleoprotein plays an important role in the transcription and replication of these viruses. The nucleoprotein encapsidates the template RNA which is the only form that is transcribed. Newly synthesized nucleoprotein seems to mediate the formation of plus-sense ribonucleoprotein complexes which are the template for synthesis of progeny minus-sense RNA (for reviews see Wagner, 1987; Kingsbury, 1991; Banerjee & Barik, 1992). In this report we present data on the expression of MBG NP in eukaryotic and prokaryotic systems and show that the mature NP is phosphorylated.

Methods

Viruses and cell lines. The Musoke strain of MBG isolated in 1980 in Kenya (Smith *et al.*, 1982) and the strain WR of vaccinia virus were propagated in E6 cells, a cloned cell line of Vero cells (ATCC CRL

† Present address: Special Pathogens Branch, Centers for Disease Control, 1600 Clifton Road, Mail Stop G14, Atlanta, Georgia 30333, U.S.A.

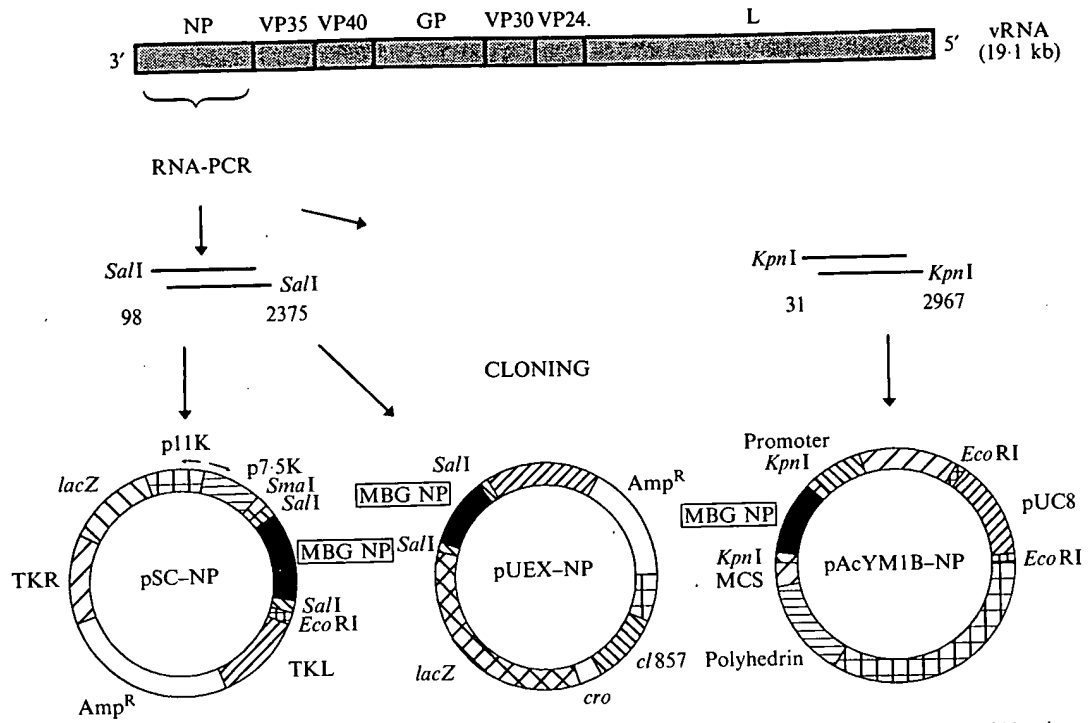

Fig. 1. Cloning strategy for the transfer plasmid vectors. The NP ORF was synthesized from genomic RNA (vRNA) using two sets of primers supplemented at their 5′ ends with either *Kpn*I or *Sal*I sites. Primer set I (*Sal*I sites) extends nucleotides 98 to 2375 and primer set II (*Kpn*I sites) nucleotides 31 to 2967. DNA fragments were cloned into the illustrated plasmid vectors: pAcYM1B for construction of recombinant baculovirus, pSC11 for construction of recombinant vaccinia virus, and pUEX for expression in *E. coli*.

1586). *Autographa californica* nuclear polyhedrosis virus (AcMNPV) was grown in a *Spodoptera frugiperda* cell line (SFVL; Kuroda *et al.*, 1986).

Propagation and labelling of virus. E6 cells were infected with MBG at an m.o.i. of 0·01. Purification of viral particles and preparation of viral antigen were performed as described previously (Mühlberger *et al.*, 1992), and the final pellet of purified virus particles was resuspended in TNE (0·01 M-Tris–HCl pH 7·4, 0·15 M-NaCl, 2 mM-EDTA) containing 1% SDS. When viral proteins were to be labelled, 20 μCi/ml [^{35}S]methionine or [^{32}P]orthophosphate (Amersham) was added 5 days post-infection (p.i.), and labelled virus was harvested 24 h thereafter. For viral genomic RNA isolation, a protocol described in detail by Mühlberger *et al.* (1992) was used.

SFVL cells were infected with AcMNPV or recombinant AcMNPV (BVNP) at an m.o.i. of 1. Virus growth (27 °C) and purification were carried out as described elsewhere (Kuroda *et al.*, 1986). For *in vivo* labelling experiments, SFVL cells were infected at an m.o.i. of 10. Forty-eight hours p.i., infected cells were starved with the appropriate nutrient-deficient medium for 2 h, labelled for 2 h by adding 100 μCi/ml [^{35}S]methionine or [^{32}P]orthophosphate, and then lysed in RIPA buffer (20 mM-MES, 55 mM-Tris–HCl, 200 mM-NaCl, 10 mM-EDTA, 1% w/v SDS pH 7·8, 1% v/v Triton X-100, 5% v/v Trasylol, 1 mM-PMSF, 10 mM-iodoacetamide). Insoluble components were pelleted at 14000 r.p.m. for 10 min in an Eppendorf centrifuge and the supernatant was stored at 4 °C.

E6 cells were infected with vaccinia virus (strain WR) or recombinant vaccinia virus (vSCNP) at an m.o.i. of 0·01. Preparation of virus stocks was performed as described by Mackett *et al.* (1986). For *in vivo* labelling experiments E6 cells were infected at an m.o.i. of 10. Twenty-four hours p.i., cells were starved with the appropriate nutrient-deficient medium for 1 h, labelled for 2 h by adding 100 μCi/ml [^{35}S]methionine or [^{32}P]orthophosphate, washed twice with PBS and lysed in RIPA buffer as described above.

Molecular cloning. The NP open reading frame (ORF) was synthesized from vRNA as described previously (Feldmann *et al.*, 1992) using the following two sets of NP-specific oligonucleotides: (i) a primer set with a *Kpn*I site (underlined), 5′ CAGGGTACCGTGTATCATATAAATAAAGAAGAATATTAAC 3′ (mRNA sense, bases 31 to 61), and 5′ CAGGGTACCGCTGCATGTATGATGAGTCCCACATTGTGA 3′ (vRNA sense, bases 2967 to 2939) and (ii) a primer set with a *Sal*I site (underlined), 5′ CAGGTCGACGAAGATATGGATTTACACAGTTTGTTGGA 3′ (mRNA, bases 98 to 126) and 5′ CAGGTCGACGACCACCATAGTATGCCCAGTCCTTGCCCG 3′ (vRNA sense, bases 2375 to 2347). The numbering is according to the genome position (for reference see EMBL Data Library, emnew: MVREPCYC; accession number Z12132). The fragment obtained by using the primer set with *Kpn*I sites was ligated into the *Kpn*I site of the plasmid vector pAcYM1B (Matsuura *et al.*, 1987). The fragment obtained by using the primer set with *Sal*I sites was ligated into the plasmid vectors pUEX (Bressan & Stanley, 1987) and pSC11 (Chakrabarti *et al.*, 1985). For cloning into pSC11, the plasmid vector was altered by introducing a *Sal*I linker into the unique *Sma*I site of the plasmid (Fig. 1). Following cloning, the entire NP ORF was sequenced in all recombinant plasmids (pUEX–NP, pAcYM1B–NP and pSC–NP) using the chain-terminating inhibitor method (Sanger *et al.*, 1977).

Expression of NP in Escherichia coli *and production of polyclonal anti-NP sera. E. coli* cells were transformed with pUEX–NP. Bacteria were grown at 30 °C and recombinant clones were selected by their ampicillin resistance. For expression studies, bacteria were grown for 5 h at 30 °C in 2YT medium containing 100 μg/ml ampicillin. Expression of the β-galactosidase–NP fusion protein (β-gal–NP) was

induced by shifting the temperature to 42 °C for 2 h. Bacteria were pelleted at 3000 r.p.m. for 10 min at 4 °C, washed once in TNE, and resuspended in a reducing lysis buffer (10% v/v glycerol, 0·187 M-Tris–HCl pH 8·8, 3% w/v SDS, 5% v/v 2-mercaptoethanol, 0·05% w/v bromophenol blue). Proteins were subjected to SDS–PAGE (Laemmli, 1970), and the gel was stained with 0·3 M-$CuCl_2$. The β-gal–NP fusion protein band was cut out and incubated in a buffer containing 250 mM-Tris–HCl pH 8·0, 1 mM EDTA for 30 min (buffer was changed three times) followed by PBS. The sample was lyophilized, ground to powder, and resuspended in PBS. The suspension was used to immunize guinea-pigs. Immunization was performed with a 1:1 (v/v) mixture of antigen and Freund's complete adjuvant (Behring). Animals were boosted six times with a 1:1 (v/v) mixture of antigen and Freund's incomplete adjuvant (Behring). Screening for antibody production was done by immunoblotting and indirect immunofluorescence assays.

Transfection of SFVL cells and selection of recombinant AcMNPV (BVNP). Transfection of SFVL cells ($2·8 \times 10^6$ cells in a six-well tissue culture plate) was performed using the lipofectin precipitation technique (Felgner *et al.*, 1987) as described by the manufacturer (Gibco BRL) with 1 μg AcMNPV wild-type DNA (Baculo Gold, Dianova) and 2 μg pAcYM1B–NP DNA. Five days post-transfection the virus titres of the supernatant fluids were determined by plaque assay as described elsewhere (Kretzschmar *et al.*, 1992). Screening for recombinant viruses was done by dot-blot hybridization according to the method of Pen *et al.* (1989) using a digoxigenin-labelled NP-specific probe (digoxigenin luminescent detection kit; Boehringer Mannheim). For this, 3×10^4 SFVL cells cultured in 96-well tissue culture plates were infected with 20 p.f.u. of virus. The supernatant fluids of positive wells were used in plaque assays in order to obtain purified recombinant BVNP (Kretzschmar *et al.*, 1992).

Construction of recombinant vaccinia virus (vSCNP). Construction of pSC–NP was performed according to the method described by Chakrabarti *et al.* (1985). For selection of recombinant virus the human cell line TK^-143 was used (Rhim *et al.*, 1975). Briefly, 1.5×10^6 TK^-143 cells were infected with vaccinia virus (WR strain) at an m.o.i. of 0·01. Cells were transfected (2 h p.i.) with 2·5 μg pSC–NP DNA by the lipofectin precipitation method (Felgner *et al.*, 1987), and 24 h post-transfection the medium was replaced with Dulbecco's medium containing 10% fetal calf serum (FCS). Forty-eight hours post-transfection cells were scraped into the medium, pelleted, resuspended in 0·5 ml Dulbecco's medium without FCS, and lysed by three cycles of freezing and thawing. This suspension was used for plaque screening on TK^-143 cells in the presence of 25 μg/ml bromodeoxyuridine (Mackett *et al.*, 1986). The plasmid vector pSC11 is constructed to express two foreign proteins, β-galactosidase under the control of the vaccinia virus 11K promoter and an additional foreign gene (NP) under the control of the 7·5K promoter (Chakrabarti *et al.*, 1985). Recombinant viruses (vSCNP), generated by homologous recombination, were plaque-purified three times on TK^-143 cells using selection for β-galactosidase-positive plaques. Forty-eight hours p.i., plaque medium containing 300 μg/ml X-Gal was added, and screening for TK^-/β-galactosidase$^+$ plaques was done 6 to 8 h later. Positive plaques (recognized by their blue colour) were further plaque-purified three times.

Dephosphorylation. Dephosphorylation of the NP was performed according to the method of Préhaud *et al.* (1990). Following *in vivo* labelling with [^{32}P]orthophosphate or [^{35}S]methionine, infected cells were lysed in a buffer containing 0·5% NP40, 20 mM-Tris-HCl and 150 mM-NaCl pH 8. One part of the cell lysate was dephosphorylated using 0·1 U/μl calf intestinal alkaline phosphatase (CIP) (Boehringer Mannheim) for 30 min at 37 °C. Controls were treated in the same way except that the CIP was omitted. After dephosphorylation, cell lysates were immunoprecipitated as described elsewhere (Huber *et al.*, 1991) using an anti-β-gal–NP guinea-pig serum (dilution 1:100) or anti-MBG guinea-pig serum (dilution 1:100) as indicated in the corresponding figures.

Immunoblot analyses. Viral proteins were separated on 10% SDS–polyacrylamide gels as described by Laemmli (1970). After electrophoresis proteins were blotted onto PVDF membranes (Millipore) by the semi-dry blot technique. Immunodetection was performed using either the polyclonal anti-β-gal–NP serum (1:300) or an anti-MBG serum raised in guinea-pigs, and a rabbit anti-guinea-pig secondary antibody coupled to horseradish peroxidase (1:500).

Indirect immunofluorescence tests. SFVL cells and E6 cells were cultured on glass coverslips and infected with AcMNPV or BVNP at an m.o.i. of 10 and vaccinia virus (WR) or vSCNP at an m.o.i. of 5 or 10, respectively. For SFVL, cells were rinsed with PBS 48 h p.i., fixed with 3% paraformaldehyde for 30 min, and the plasma membranes were lysed for 30 min in 2% Triton X-100 in PBS (containing calcium and magnesium). E6 cells were rinsed with PBS and fixed with cold acetone (−20 °C) for 10 min. After fixation, cells were incubated for 1 h at room temperature with different primary antibodies. For vSCNP-infected E6 cells this was an anti-MBG guinea-pig serum, diluted 1:40 in PBS, and for BVNP-infected SFVL cells an anti-β-gal–NP guinea-pig serum, diluted 1:40 in PBS. Subsequently, cells were washed three times with PBS and incubated with a fluorescein isothiocyanate-conjugated rabbit anti-guinea-pig antiserum (Dako) diluted 1:50 in PBS, for 1 h at room temperature. Finally the cells were washed three times with PBS and examined using a fluorescence microscope (Zeiss, Axiomat).

Results

PCR amplification and cloning of the entire NP ORF

The entire ORF of the NP gene of MBG strain Musoke was synthesized from genomic RNA (vRNA) by RNA PCR using two sets of specific oligonucleotides (Fig. 1). The NP ORF starting with the AUG codon at positions 104 to 106 and terminating at positions 2189 to 2191 (UAG) is 2088 nucleotides long and encodes a protein of 695 amino acids with a predicted M_r of 77·86K (Sanchez *et al.*, 1992). The DNA fragment synthesized using the primer pair with *Kpn*I sites had a length of 2937 nucleotides (positions 31 to 2967) and contained a part of the 3′ untranslated region of the genome (16 nucleotides), the entire NP gene including the transcription initiation and termination signals (positions 47 to 2843), the NP–VP35 intergenic region, and part of the VP35 gene (positions 2851 to 2967). This fragment was cloned into the plasmid vector pAcYM1B under the control of the polyhedrin promoter using the *Kpn*I site of the multiple cloning site (MCS). The DNA fragment produced using the primer pair with the *Sal*I sites had a length of 2278 nucleotides (positions 98 to 2375) and contained the last four nucleotides of the 3′ non-coding region of the NP gene, the NP ORF, and 184 nucleotides of the 5′ non-coding region of the NP gene (transcription signals are not included) (Sanchez *et al.*, 1992; Feldmann *et al.*, 1992). This fragment was cloned into the plasmid vector pUEX using the *Sal*I site of the MCS and the

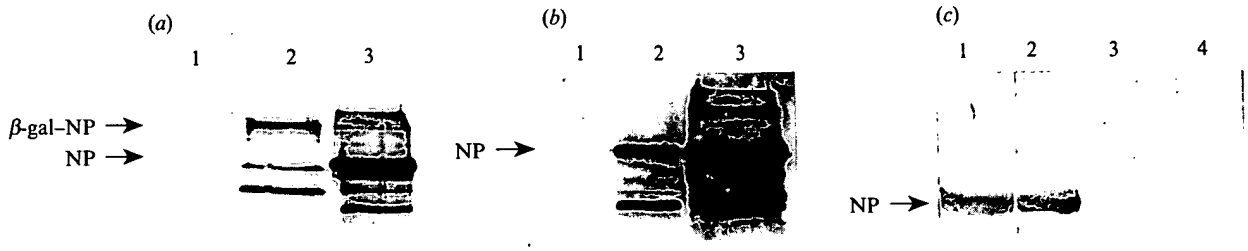

Fig. 2. Expression of recombinant NP. Infection of cells, cell lysis, electrophoresis and immunoblotting were performed as described in Methods. Blots were probed with a guinea-pig anti-MBG serum, diluted 1:300. Bound antibody was detected with peroxidase-conjugated rabbit anti-guinea-pig Ig. (*a*) Immunoblot analysis of *E. coli* transformed with pUEX–NP. Lane 1, *E. coli* only; lane 2, pUEX–NP-transformed *E. coli*; lane 3, MBG virion proteins. (*b*) Immunoblot analysis of vSCNP-infected E6 cells. Lane 1, vaccinia virus (strain WR)-infected cells; lane 2, vSCNP-infected cells; lane 3, MBG virion proteins. (*c*) Immunoblot analysis of BVNP-infected SFVL cells. Lane 1, BVNP-infected SFVL cells; lane 2, MBG virion proteins; lane 3, AcMNPV (wild-type) infection; lane 4, mock infection.

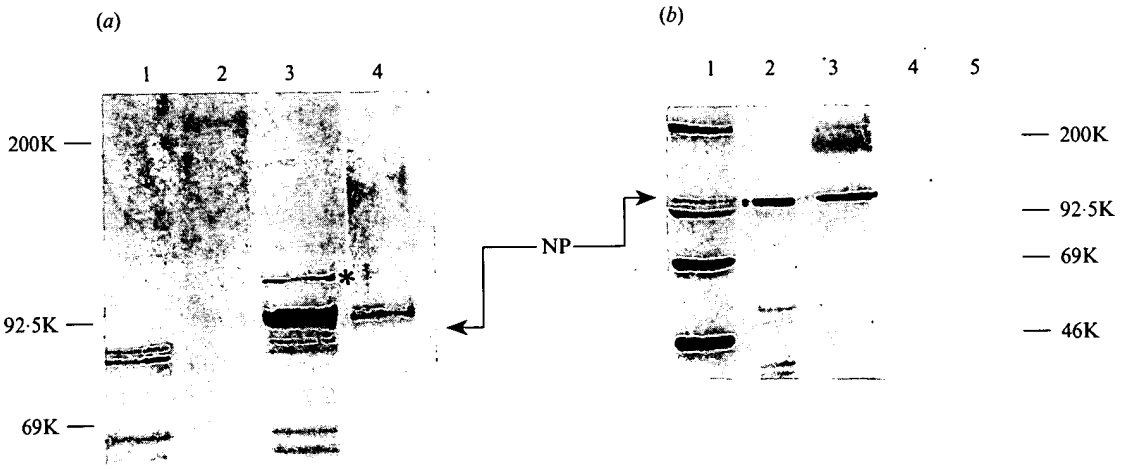

Fig. 3. *In vivo* [^{35}S]methionine labelling of recombinant NP and mature virion NP. Infection of cells, *in vivo* labelling, cell lysis and preparation for SDS–PAGE were done as described in Methods. (*a*) E6 cells infected with vSCNP. Cell lysates were immunoprecipitated using an anti-MBG guinea-pig serum (dilution 1:100). Lane 1, vaccinia virus (strain WR)-infected cells; lane 2, mock infection; lane 3, vSCNP-infected cells [asterisk marks β-galactosidase (background) which is expressed only by the recombinant virus; see Methods]; lane 4, MBG virion proteins. (*b*) SFVL cells infected with BVNP. Cell lysates were immunoprecipitated using an anti-β-gal–NP guinea-pig serum (dilution 1:100). MBG virion proteins were not immunoprecipitated. Lane 1, M_r markers (Amersham); lane 2, BVNP infection; lane 3, MBG virion proteins; lane 4, AcMNPV (wild-type) infection; lane 5, mock infection.

plasmid vector pSC11 under the control of the early/late 7·5K promoter. Sequence analysis of the recombinant plasmids revealed no deletions, insertions or point mutations in the cloned fragments, and confirmed the correct orientation in the appropriate vectors (data not shown).

Expression of recombinant NP

Expression from the pUEX plasmid is controlled by the *cI*857 repressor gene, whose temperature-sensitive protein product shuts off the P_r promoter. Expression is achieved by transferring the host cells from the normal growth temperature (30 °C) to 42 °C. In this system, the NP was expressed as an insoluble fusion protein in which the fusion point was close to the C terminus of the β-galactosidase. The fusion protein had a predicted M_r of approximately 200K and migrated on SDS–polyacrylamide gels as expected. In immunoblot analysis, the fusion protein reacted specifically with an anti-MBG guinea-pig serum demonstrating that the NP ORF was expressed as a part of the fusion product (Fig. 2*a*). The fusion product β-gal–NP was easily separated by SDS–PAGE from other *E. coli* proteins owing to its high M_r. It was used in a polyacrylamide gel suspension for immunization of guinea-pigs. After six immunization

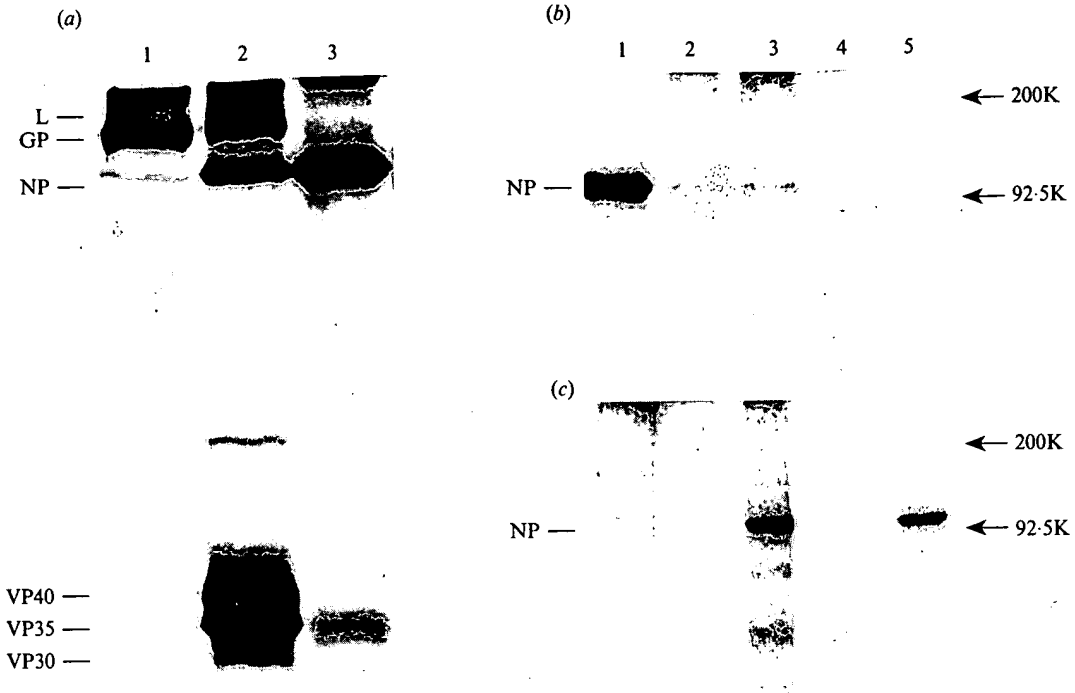

Fig. 4. *In vivo* [^{32}P]orthophosphate labelling of recombinant NP and mature virion NP. Infection of cells, *in vivo* labelling, cell lysis, dephosphorylation and preparation for SDS–PAGE were performed as described in detail in Methods. (*a*) Phosphorylation of virion structural proteins. Radiolabelled purified virion particles were lysed and the proteins were subjected to SDS–PAGE. Lane 1, [^{3}H]glucosamine-labelled MBG virion proteins; lane 2, [^{35}S]methionine-labelled MBG virion proteins; lane 3, [^{32}P]orthophosphate-labelled MBG virion proteins. (*b*) Phosphorylation of NP in E6 cells infected with vSCNP. Cell lysates were immunoprecipitated using an anti-MBG guinea-pig serum (dilution 1:100). Lane 1, MBG virion proteins; lanes 2 and 3, vSCNP infection; lane 4, vaccinia virus (strain WR) infection; lane 5, mock infection (*c*) Phosphorylation of NP in SFVL cells infected with BVNP. Cell lysates were immunoprecipited using an anti-β-gal–NP guinea-pig serum (dilution 1:100). Lane 1, AcMNPV (wild-type) infection; lane 2, BVNP infection and treatment with alkaline phosphatase (CIP); lane 3, BVNP infection untreated; lane 4, MBG virion proteins treated with CIP; lane 5, MBG virion proteins untreated.

boosts the sera reacted positively in immunoblot analyses to the mature viral NP as well as to recombinant NP with titres of 1/1000 to 1/2000. These polyclonal anti-β-gal–NP sera were used at dilutions of 1:40 in indirect immunofluorescence tests, 1:100 in immunoprecipitation assays and 1:300 in immunoblot analyses.

Expression of the NP ORF in both mammalian cells (E6), by recombinant vaccinia virus (vSCNP), and insect cells (SFVL), by recombinant baculovirus (BVNP), resulted in a protein product of the expected M_r (Kiley *et al.*, 1988; Sanchez *et al.*, 1992) migrating in SDS–PAGE as a 94K protein and comigrating with the mature viral NP. Immunoblots with lysates of BVNP-infected SFVL and vSCNP-infected E6 cells using the monospecific anti-β-gal–NP sera confirmed the expression of MBG NP in both systems (Fig. 2*b* and *c*). Additional protein bands of lower M_r, which also reacted with the sera used for immunoblot analyses represent degradation products of the NP and were also commonly seen in preparations of MBG structural proteins (Fig. 2*a* and *b*, lanes 3). BVNP-infected SFVL cells expressed high levels of recombinant NP constituting approximately 10% of the total cellular protein mass. The protein could be visualized by Coomassie blue staining as shown in Fig. 5(*b*). In contrast, the level of NP expression in E6 cells did not allow detection by Coomassie blue staining.

The expression of recombinant NP could also be shown by *in vivo* labelling experiments in both eukaryotic systems using [^{35}S]methionine (Fig. 3*a* and *b* and Fig. 5*a*). In both expression systems (vaccinia virus and baculovirus) the recombinant NP appeared as a double band in SDS–PAGE. The lower mobility band had an apparent M_r of 94K and comigrated with the *in vivo* [^{35}S]methionine-labelled mature viral NP (Fig. 3*a*,*b* and Fig. 5*a*). The second band had a slightly increased mobility on SDS–PAGE, migrated with an M_r of approximately 92K and was not present in viral particles (Fig. 3*a*,*b* and Fig. 5*a*). These two forms were separated only when the amount of the protein loaded was low and could be also detected by Coomassie blue staining in the case of BVNP-infected SFVL cells (Fig. 5*b*). Both proteins reacted with the polyclonal anti-β-gal–NP sera. Infection of E6 cells with vSCNP resulted in coexpression of β-galactosidase due to the expression vector used for

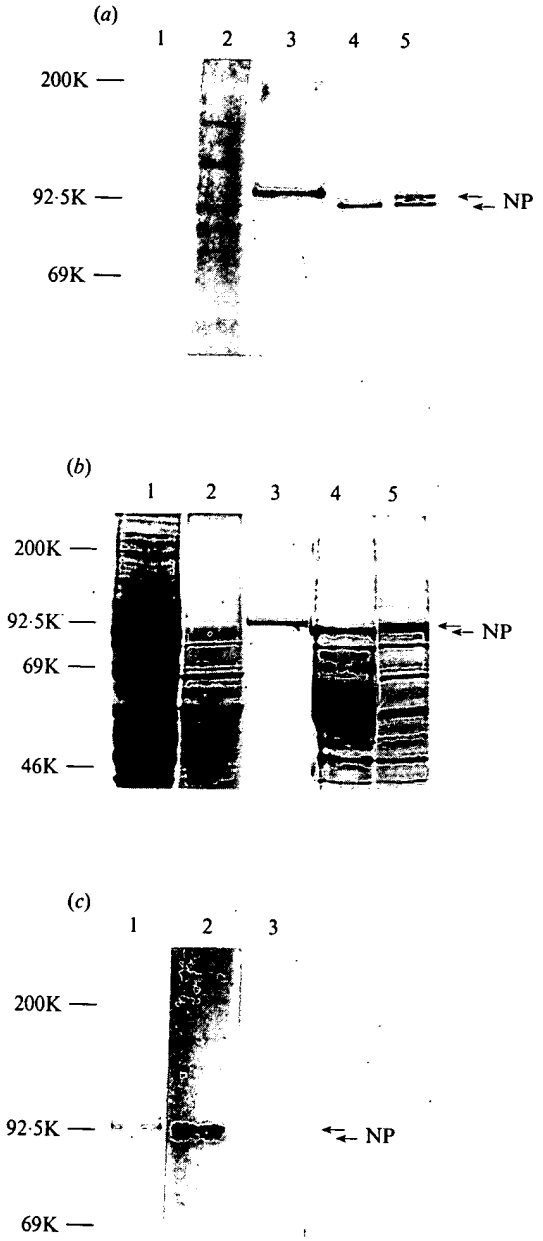

Fig. 5. Two different forms of the NP. (*a*) Immunoprecipitation of [^{35}S]methionine-labelled recombinant NP. Infection of insect cells and treatment of the SFVL cell-derived recombinant NP with alkaline phosphatase (CIP) was done as described in Methods. Cell lysates were immunoprecipitated using an anti-β-gal–NP guinea-pig serum (dilution 1:100). Lane 1, AcMNPV (wild-type) infection; lane 2, mock infection; lane 3, MBG virion proteins; lane 4, BVNP-infected SFVL cells, treated with CIP; lane 5, BVNP-infected SFVL cells, untreated. (*b*) Coomassie blue-stained gel. Lane 1, mock infection; lane 2, AcMNPV (wild-type) infection; lane 3, MBG virion proteins; lane 4, BVNP-infected SFVL cells, treated with CIP (the heavily stained protein represents the CIP); lane 5, BVNP-infected SFVL cells, untreated. (*c*) Immunoprecipitation of [^{35}S]methionine-labelled MBG-infected E6 cells. Lane 1, MBG virion proteins; lane 2, MBG-infected cells; lane 3, mock infection.

generating the recombinant virus (Fig. 3*a*, lane 3, marked by asterisk).

Phosphorylation of the NP

Propagation of MBG in E6 cells in the presence of [^{32}P]orthophosphate revealed two phosphorylated virus structural proteins (Fig. 4*a*). The major phosphorylated protein comigrated with the ^{35}S-labelled viral NP. The second, weakly phosphorylated, protein had an apparent M_r of 30K to 35K and could be either VP35 or VP30. This finding is in line with the observation made for EBO which demonstrated phosphorylation of NP and VP30 (Elliott *et al.*, 1985). *In vivo* labelling experiments with vSCNP-infected E6 cells and BVNP-infected SFVL cells using [^{32}P]orthophosphate confirmed the phosphorylation of the NP. In contrast to the double band that was detected by [^{35}S]methionine labelling, a single labelled protein was immunoprecipitated which comigrated with the mature *in vivo* ^{32}P-labelled virion NP (Fig. 4*b* and *c*). CIP treatment of the immunoprecipitated [^{32}P]orthophosphate-labelled proteins revealed loss of the label (Fig. 4*c*) whereas immunoblot analysis proved that the protein was not degraded by the treatment (data not shown).

Two different forms of NP

The two forms of the NP, which were found in cells expressing the recombinant NP, could also be detected in MBG-infected E6 cells by *in vivo* [^{35}S]methionine labelling (Fig. 5*c*, lane 2). In order to investigate the nature of these two forms, BVNP-infected insect cells were labelled with [^{35}S]methionine and the proteins were then dephosphorylated with CIP. Phosphatase treatment resulted in the loss of the 94K form of NP (Fig. 5*a*, lanes 4 and 5, see arrows) and an increase in the intensity of the 92K form. Fig. 5(*b*) shows a Coomassie blue-stained SDS–polyacrylamide gel of BVNP-infected SFVL cell lysates treated in the same manner as described for Fig. 5(*a*) and again it can be seen that the 94K form was converted into the 92K form. The heavily stained protein in lane 4 with an M_r of 69K represents the CIP. These data demonstrated that the NP exists in two forms, a phosphorylated 94K form and an unphosphorylated 92K form. However, in virion particles (Fig. 5*a* and *b*, lanes 3) the NP is present only in the phosphorylated (94K) form.

Intracellular distribution of the recombinant NP

Indirect immunofluorescence assays using an anti-MBG guinea-pig serum (E6 cells) or the anti-β-gal–NP serum (insect cells) were performed to determine the intracellular distribution of the NP expressed in mammalian and insect cells. Both recombinant NPs were exclusively

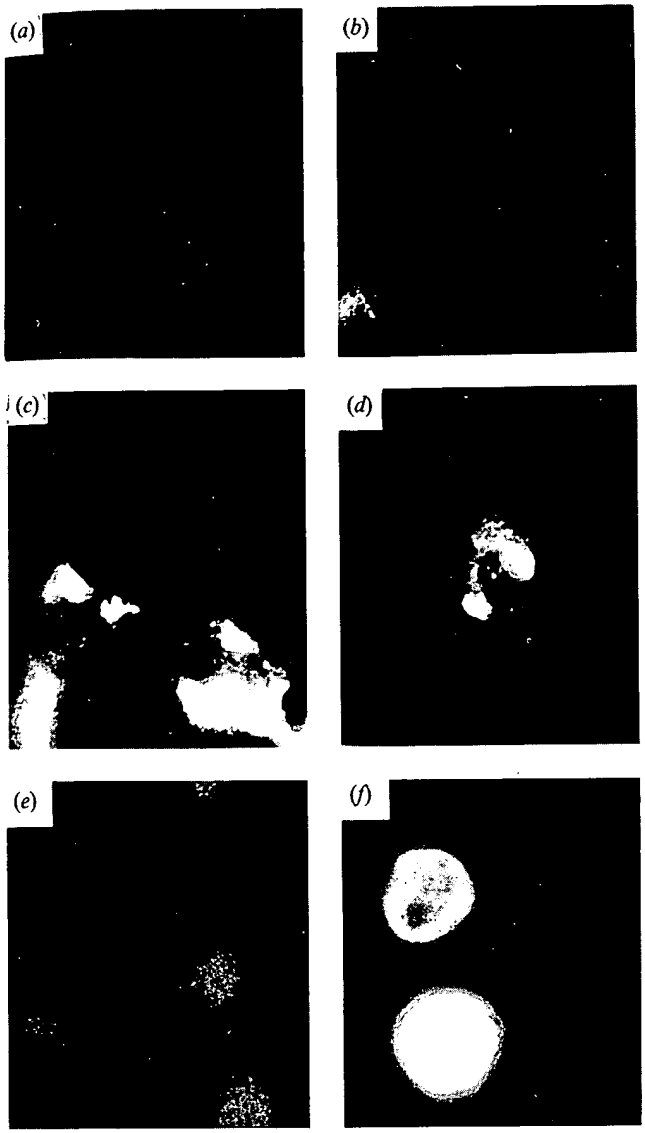

Fig. 6. Localization of NP in infected cells by indirect immunofluorescence assay. Infection of cells and detection of expressed recombinant NP using an anti-MBG guinea-pig serum (dilution 1:40) (vaccinia virus) and an anti-β-gal–NP guinea-pig serum (baculovirus) were done as described in Methods. (*a*) Vero E6 cells, mock infection; (*b*) Vero E6 cells, vaccinia virus (strain WR) infection; (*c*) and (*d*) Vero E6 cells, vSCNP infection; (*e*) SFVL cells, AcMNPV (wild-type) infection; (*f*) SFVL cells, BVNP infection.

localized in the cytoplasm of the infected cells. vSCNP-infected E6 cells showed large, primarily perinuclear, cytoplasmic inclusion bodies (Fig. 6*a* to *d*) whereas SFVL cells infected with BVNP showed a uniform distribution of the NP in the cytoplasm (Fig. 6*e* and *f*).

Discussion

Sequence analysis of the genes encoding filovirus NPs and comparison with the sequences of other NNS RNA virus nucleoproteins suggests that filovirus NP seems to be the functional analogue of the nucleoproteins of paramyxoviruses and rhabdoviruses (Sanchez *et al.*, 1992). This is further supported by the fact that filoviral NP genes are located at the extreme 3′ end of the viral genome as found with all NNS RNA virus nucleoprotein genes (Feldmann *et al.*, 1992). As shown for all nucleoproteins, filoviral NPs are the major components of the viral ribonucleoprotein complex and are tightly bound within the complexes. However, in comparison to all other NNS RNA viruses, filoviral NPs have unusually high M_rs. Whereas the nucleoproteins of paramyxoviruses range from 42K to 62K (Morgan, 1991) and those of rhabdoviruses from 47K to 62K (Nichol, 1993), the NP of MBG has an M_r of 94K and that of EBO an M_r of 104K (Elliott *et al.*, 1985). This fact suggests that filoviral NPs may have an additional function compared with the nucleoproteins of paramyxoviruses and rhabdoviruses and this function may be located in the C-terminal part of the NP.

In order to characterize filovirus NPs better and to define their functional role we expressed the MBG (strain Musoke) NP in different cell systems. The vaccinia virus system was chosen because of its ability to infect Vero cells, a mammalian cell line that is commonly used for the propagation of all filoviruses and thus reflects more authentic conditions. The baculovirus system was chosen for expression of the protein in insect cells (SFVL cell line); this is known to provide large amounts of expressed foreign proteins (Luckow & Summers, 1988). The recombinant proteins were examined for their cellular localization and co- and post-translational modifications. In addition, the MBG NP was expressed as a C-terminal β-galactosidase fusion protein in *E. coli* for production of specific anti-NP sera as tools for diagnosis and research.

Expression of the MBG NP in both cell lines revealed a product of the expected M_r that comigrated on SDS–PAGE with the mature viral NP (Fig. 2 to 5). As expected, NP was expressed to higher levels in insect cells infected with the recombinant baculovirus (BVNP) (Fig. 5*b*) than in E6 cells infected with the recombinant vaccinia virus (vSCNP). Expression of NP by vSCNP could only be shown by immunoblot analysis or *in vivo* labelling (Fig. 2*b* and 3*a*). This phenomenon may be due in part to the fact that the NP ORF contains a vaccinia virus early transcription termination signal (3′ TTTTTAT 5′; Rohrmann *et al.*, 1986) 393 nucleotides downstream of the AUG initiation codon (Sanchez *et al.*, 1992).

In vivo labelling of virion particles using [^{32}P]orthophosphate in combination with CIP treatment has clearly demonstrated that the mature MBG NP is phosphorylated (Fig. 4). Phosphorylation is not a unique feature of filoviral NPs. Among NNS RNA viruses the

nucleoproteins of rabies virus (Sokol & Clark, 1973), Sendai virus (Lamb & Choppin, 1977) and measles virus (Norrby & Oxman, 1990) are phosphorylated.

Phosphorylation of NP was also demonstrated in both expression systems investigated (Fig. 4). This indicates that phosphorylation of MBG NP could be mediated in part by cellular kinases. Acceptors for phosphorylation can be either serine, threonine or tyrosine residues depending on the particular protein kinase (for review see Leader & Katan, 1988). These amino acid residues are found throughout the MBG NP. Previous studies have shown that different phosphorylated residues on a single protein result from the action of different protein kinases. This was shown for glycogen synthetase (Cohen, 1985) and the phosphorylation of the P protein of vesicular stomatitis virus which is carried out by both cellular kinases and the kinase activities of the viral L protein (Banerjee & Barik, 1992). Whether phosphorylation of NP during MBG infection is performed in part by the viral L protein is a subject of current investigations. ATP-binding site motifs have been identified in the MBG L protein sequence indicating the possibility of a kinase function for this large protein (Mühlberger *et al.*, 1992). For Sendai virus, Einberger *et al.* (1990) have demonstrated a kinase activity of the L protein by *in vitro* phosphorylation of NP and P protein. In the case of the recombinant MBG NP one should also consider the possibility of kinase activities related to an infection with baculovirus or vaccinia virus. Therefore our studies do not necessarily allow the conclusion that phosphorylation of recombinant NP is due to the same enzymatic activities and the use of identical acceptors for phosphorylation as in MBG-infected cells.

Expression of the NP ORF in both systems as well as in the MBG-infected E6 cells revealed the synthesis of a larger phosphorylated and a smaller unphosphorylated form of the protein (difference in M_r approx. 2K). However, in virion particles only the phosphorylated (94K) form could be detected (Fig. 3 to 5). A similar phenomenon was observed when recombinant simian virus 40 large T antigen was expressed in human 293 cells. Expression resulted in two forms of the large T antigen differing in M_r by 1K which was due to a conformational change related to phosphorylation of threonine residues (Grässer & König, 1992). On the other hand, the lower M_r form could also be the result of a proteolytic process as shown for the major nucleocapsid protein of influenza virus. This appears, depending on the cell type, at late stages of the infection cycle in two differently migrating forms (Zhirnov & Bukrinskaya, 1981). NP_{53} (53K) was shown to be the proteolytically cleaved form of NP_{56} (56K). However, the fact that [^{32}P]orthophosphate labelling of the NP showed only the larger form to be phosphorylated and the conversion of the 94K form into the 92K form by phosphatase treatment do not support proteolytic degradation.

Indirect immunofluorescence studies demonstrated that recombinant NP is found exclusively in the cytoplasm of infected cells (Fig. 6). The immunostaining pattern which can be seen in these cells is similar to the pattern found in MBG-infected Vero cells and human endothelial cells (Becker *et al.*, 1992; Schnittler *et al.*, 1993). In MBG-infected cells this pattern is related to the formation of intracytoplasmic inclusion bodies which consist of viral nucleocapsids. Since viral nucleocapsids are composed of genomic RNA, NP and probably three additional viral proteins (VP35, VP30 and L) (Elliott *et al.*, 1985; Kiley *et al.*, 1988), our findings indicate that nucleocapsid-like structures can also be formed by spontaneous aggregation of NP molecules. Furthermore, NP molecules could also aggregate with cellular RNAs and/or proteins resulting in formation of nucleocapsid-like structures. Formation of spontaneous aggregates was also found in cells expressing recombinant nucleocapsid protein of vesicular stomatitis virus (Sprague *et al.*, 1983) or measles virus nucleoprotein (Spehner *et al.*, 1991).

The exact function of phosphorylation of the nucleoproteins of NNS RNA viruses has not yet been elucidated. It is noteworthy that only the phosphorylated form of the NP is found in virion particles of MBG. This result may indicate that only the phosphorylated NP is able to interact with genomic RNA and form the nucleocapsid complex. It is known for several proteins that phosphorylation is a prerequisite for binding to RNA. The rex protein of human T lymphotropic virus type II loses its affinity for the R region of the 5′ long terminal repeat after treatment with phosphatases (Green *et al.*, 1992). Han *et al.* (1992) showed that phosphorylation determines the interaction between the trans-activation response (TAR) region of human immunodeficiency virus type 1 and a cellular TAR RNA stem-binding factor. In this context phosphorylation of the NP should be considered in relation to the role of this protein during the transcription and replication of MBG.

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SeV infection. Furthermore the VSV M
without using a virus vector was
eased into the culture medium. These results suggest
M protein has a budding activity per se and that
are passively involved in the release of VSV M.
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Accommodation of foreign genes into the Sendai virus genome: sizes of inserted genes and viral replication

THOR(S): Sakai, Yuko; Kiyotani, Katsuhiro; Fukumura, Masayuki; Asakawa, Makoto; Kato, Atsushi; Shioda, Tatsuo; Yoshida, Tetsuya; Tanaka, Akemi; Hasegawa, Mamoru; Nagai, Yoshiyuki

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AB Sendai virus (S V) is an enveloped virus with a neg. sense genome RNA of about 15.3 kb. We previously established a system to recover an infectious virus entirely from SeV cDNA and illustrated the feasibility of using SeV as a novel expression vector. Here, we have attempted to insert a series of foreign genes into SeV of different lengths to learn how far SeV can accommodate extra genes and how the length of insert genes affects viral replication in cells cultured in vitro and in the natural host, mice. We show that a gene up to 3.2 kb can be inserted and efficiently expressed and that the ...ication speed as well as the final virus titers in cell culture ...d as the inserted gene length increases. ... very clear but a ... generally

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Accommodation of foreign genes into the Sendai virus genome: sizes of inserted genes and viral replication

Yuko Sakai[a], Katsuhiro Kiyotani[c], Masayuki Fukumura[d], Makoto Asakawa[d], Atsushi Kato[a], Tatsuo Shioda[b], Tetsuya Yoshida[c], Akemi Tanaka[e], Mamoru Hasegawa[d], Yoshiyuki Nagai[a,*]

[a] *Department of Viral Infection, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo, Japan*
[b] *Department of Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo, Japan*
[c] *Department of Bacteriology, Hiroshima University School of Medicine, Hiroshima, Japan*
[d] *DNAVEC Research, Tsukuba, Japan*
[e] *Department of Pediatrics, Osaka City University Medical School, Osaka, Japan*

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Abstract Sendai virus (SeV) is an enveloped virus with a negative sense genome RNA of about 15.3 kb. We previously established a system to recover an infectious virus entirely from SeV cDNA and illustrated the feasibility of using SeV as a novel expression vector. Here, we have attempted to insert a series of foreign genes into SeV of different lengths to learn how far SeV can accommodate extra genes and how the length of inserted genes affects viral replication in cells cultured in vitro and in the natural host, mice. We show that a gene up to 3.2 kb can be inserted and efficiently expressed and that the replication speed as well as the final virus titers in cell culture are proportionally reduced as the inserted gene length increases. In vivo, such a size-dependent effect was not very clear but a remarkably attenuated replication and pathogenicity were generally seen. Our data further confirmed reinforcement of foreign gene expression in vitro from the V(−) version of SeV in which the accessory V gene had been knocked out. Based on these results, we discuss the utility of SeV vector in terms of both efficiency and safety.

Key words: Sendai virus; Genome; Replication

1. Introduction

Sendai virus (SeV), a member of the genus *Respirovirus* in the family *Paramyxoviridae*, is an enveloped virus with a non-segmented negative sense genome RNA of about 15.3 kb. In the genome RNA of negative sense (−)RNA, the extracistronic 3′ leader and 5′ trailer regions, which are about 50 nucleotides in length and contain *cis*-acting elements essential for replication, flank six genes, which encode the nucleocapsid (N) protein, phospho (P) protein, matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN) and large (L) protein in this order from the 3′ terminus. The genome RNA is tightly associated with the structural subunits, N proteins, and further complexed with the RNA polymerase comprising the L and P proteins [1], forming a helical ribonucleoprotein complex, (−)RNP. L represents the catalytic chain and P is an essential modulator in RNA synthesis. The (−)RNP complex, not the naked RNA, is functional as the template for both transcription and replication. There is only a single promoter at the 3′ end for the polymerase. By recognizing the end (E) (termination/polyadenylation) and start (S) signals present at each gene boundary, the polymerase gives rise to each mRNA (reviewed in [2,3]). After translation of these mRNAs and accumulation of the translation products, genome replication begins. Here, the same polymerase copies the same RNP template, but now ignores the successive stop signals and reads through each gene boundary to generate a full length antigenomic (+)RNP. Association of the newly synthesized N subunits with the nascent RNA chain is a precondition for read-through. Thus, encapsidation and replication are tightly coupled and synchronized with each other. The (+)RNP, in turn, serves as the template for the synthesis of (−)RNP. The viral components meet at the plasma membrane and the mature virions are formed and released by budding.

We have established a system to recover SeV entirely from cDNA with a remarkably high efficiency [4]. Thus, the SeV genome can be changed at will and the outcomes can be evaluated in the context of not only viral replication in culture cells but also viral pathogenesis in the natural host, mice ([5–8], for a review see [3]). The technology has also opened the possibility to use SeV as a novel expression vector ([9–11], for a review see [3]). SeV is only moderately pathogenic for cells in culture, reaches an extremely high copy number in cells and has a broad cellular host range. Moreover, the V(−) SeV whose V protein expression was knocked out reached even a higher copy number than the standard SeV [5,6] and is therefore extremely useful to achieve a high level of foreign gene expression in cells of interest. For instance, the expression level of gp120 envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1) from the V(−) version in certain cell lines appeared to be the highest available in mammalian cells [10]. Thus, recombinant SeV technology is greatly facilitating biochemical and structural studies of medically important proteins ([12], for a review see [3]). The SeV V protein itself is an accessory protein. Although perfectly competent for tissue culture replication as described above, the V knock out SeV was found to be rapidly cleared from the mouse lung by some innate immunity recruited early in infection and severely impaired in virulence for mice, suggesting that the V protein encodes a luxury function required for in vivo pathogenesis ([5,6], for a review, see [3]).

In this study, we attempted to insert into SeV and express from SeV foreign genes of various lengths to know how far SeV can tolerate additional length and how viral replication both in vitro and in vivo is affected by the length. The results indicated that SeV can stably express genes up to 3.2 kb in length. The viral replication in cell culture was retarded conversely as the inserted gene length was increased. Viral multi-

*Corresponding author. Fax: (81) (3) 5449-5409.
E-mail: ynagai@ims.u-tokyo.ac.jp

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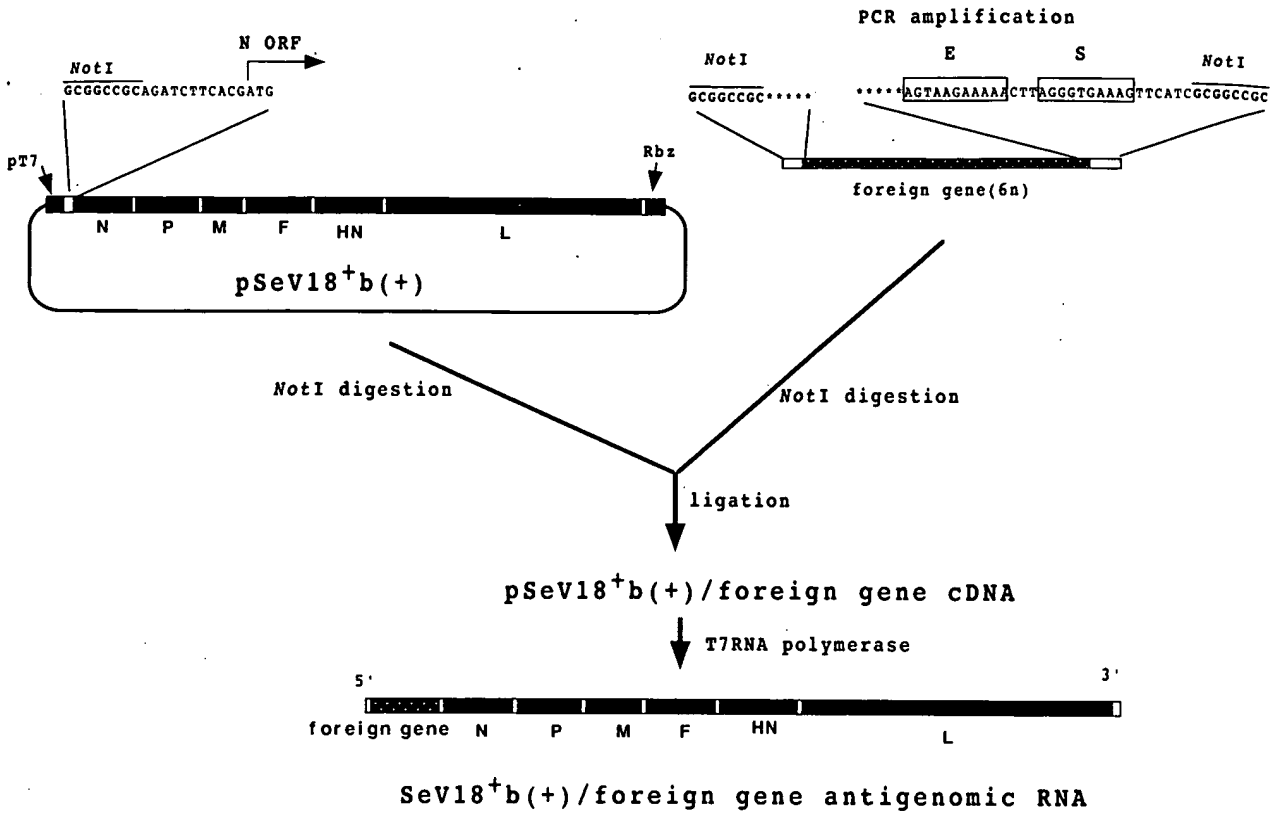

Fig. 1. Insertion of a foreign gene into the SeV cDNA plasmid. The SeV cDNA plasmid, named pSeV18$^+$b(+), contains an 18 nucleotide insert with a unique *Not*I site just upstream of the N ORF within the 3′ proximal N gene and generates antigenomic positive sense RNA. This construct allowed for a straightforward insertion of a gene PCR-amplified with *Not*I-tagged primers in a cassette-like fashion. SeV specific transcription termination (E) and restart (S) signals connected with a trinucleotide intergenic sequence were included in the antisense primer for the PCR reaction. Not only the standard SeV but also the V(−) version were used (see the text).

plication and pathogenicity in mice were also attenuated. The results confirmed the superiority of the V(−) version for a higher expression as well as convenience of the cassette-like insertion developed previously [9].

2. Materials and methods

2.1. Insertion of foreign genes into SeV cDNA plasmid

Fig. 1 illustrates the basic strategies for insertion of a foreign gene into the SeV genome. The SeV genome length appears to have to be a multiple of six nucleotides for efficient replication [13]. Without violating this 'rule of six', the SeV cDNA plasmid, named pSeV18b(+), was constructed, which contains an 18 nucleotide insert with a unique *Not*I site just upstream of the N open reading frame (ORF) within the 3′ proximal N gene (in genomic negative sense) and generates antigenomic positive sense RNA [9] (Fig. 1). This construct allowed for a straightforward insertion of a gene PCR-amplified with *Not*I-tagged primers (again without violating the rule of six) in a cassette-like fashion [9]. SeV specific transcription termination (E) and restart (S) signals connected with a trinucleotide intergenic sequence were included in the antisense primer for the PCR reaction. The recovered virus was named SeV18b+. Using the synthetic E and S signals, the inserted gene expression is terminated and the downstream neighbor N gene expression is initiated, respectively (Fig. 1). The transcription start of the inserted gene itself is driven by the S signal originally used for the N gene start of the parental SeV. In SeV transcription, restart at each gene boundary is efficient but not perfect, causing polar attenuation of gene expression toward the 5′ end (reviewed in [2]). All insertions were therefore made in the upstream region of the 3′ proximal N gene ORF. Not only the standard SeV but also the V(−) version were used for a higher level of expression [10]. The foreign genes, which we attempted to insert, included those encoding stromal cell-derived factor 1α (SDF-1α) [11], green fluorescent protein of *Aequore victoria* (GFP), HIV-1 gp120 [10], firefly luciferase (luci) [9], β-glucuronidase (β-glu) and *lacZ* encoding β-galactosidase (β-gal). The sizes of these foreign genes ranged from 0.4 to 3.2 kb (Table 1). β-Glu is relevant to mucopolysaccharidosis VII.

2.2. Transfection of cDNAs and infectious virus recovery

Viruses were recovered from cDNAs essentially according to the previously described procedure [4]. Briefly, 1.2×10^7 LLCMK2 cells, a monkey kidney line, were infected with a recombinant vaccinia virus (VV), vTF7-3, expressing T7 polymerase [14] at a multiplicity of two plaque forming units (PFU) per cell. Then, 60 μg of a SeV plasmid with a given insert and the plasmids encoding *trans*-acting proteins, pGEM-N (24 μg), pGEM-P (12 μg) and pGEM-L (24 μg), were transfected simultaneously with aid of the lipofection reagent DOTAP (Boehringer-Mannheim). The cells were maintained in serum free minimal essential medium in the presence of 40 μg/ml cytosine arabinofuranoside and 100 μg/ml rifampicin to minimize VV cytopathogenic-

Table 1
Sizes of inserted genes and the final titers of recovered recombinant SeVs after propagation in embryonated chicken eggs

Inserted gene	Size (kb)	Titer (10^9 PFU/ml)	
		SeV18$^+$b	V(−) SeV18$^+$b
18$^+$b		7.93	4.33
SDF-1α	0.4	9.74	4.79
GFP	0.7	1.14	4.47
HIV gp120	1.6	0.39	2.45
Luci	1.7	3.03	–
β-Glu	2.1	0.74	–
β-Gal	3.2	2.79	1.62

–, not done.

ity and thereby maximize the recovery rate [4]. 40 h after transfection, cells were harvested, disrupted by three cycles of freezing and thawing and injected into the allantoic cavity of 10 days old embryonated chicken eggs. After 3 days of incubation, the allantoic fluid was harvested. The titers of recovered viruses were expressed in hemagglutination units (HAU) and PFU per ml as described previously [4]. The allantoic fluid of the eggs contained 10^8–10^9 PFU per ml of the recovered virus along with the helper VV in an amount of 10^3–10^4 PFU per ml. The latter was completely eliminated by the second propagation in eggs at a dilution of 10^{-6} [4]. The second passages were used as the stock viruses for all the experiments.

2.3. Infection and pathogenicity studies

CV1 cells, a monkey kidney cell line, were used for studies of tissue culture replication as described previously [5,6]. For in vivo replication and pathogenicity studies, specific pathogen free, 3 weeks old mice of the strain ICR/Crj (CD-1) (Charles River, Tokyo, Japan) were used. Virus titers in the lungs were determined by cell infectious units per lung and pathogenicity by consolidation scores of the lungs and disturbance of body weight gain as described [5,6,8].

3. Results

3.1. Recovery of recombinant viruses

We inserted various foreign genes into the cDNAs encoding the standard or V(−) SeV antigenome RNA or both and attempted virus recovery according to the protocol described in Section 2 and illustrated in Fig. 2. The *Not*I-based cassette insertion has been generally convenient as no *Not*I site was present in the genes to be inserted except *lacZ*. One *Not*I site was present in the *lacZ*. This one was disrupted prior to PCR amplification. Virus recovery was successful in most attempts. However, there were some but rare unsuccessful cases despite repeated trials. The reason for this was initially unclear but careful inspection of the sequences to be inserted revealed that these genes contained a region resembling the SeV E signal (AUUCUUUUU). Most likely, such an E-like sequence induced premature transcription termination, eventually leading to failure of virus recovery. In these cases, E-like sequences were disrupted and viruses were successfully recovered. The final titers of recovered recombinants after propagation in embryonated chicken eggs are shown in Table 1.

We previously showed that the recombinant SeV18b+ with an 18 nucleotide insert and the original wild-type SeV were very similar in replication in cultured cells and multiplication and pathogenicity in mice. SeV18b+ but not wild-type cDNA was the starting material for insertion of foreign genes (see Section 2). Thus, we used SeV18b+ or its V(−) version as a control for all subsequent experiments.

Fig. 2 compares replication kinetics of various recombinant viruses derived from the standard V(+) version under the conditions of both a single round and multiple rounds of replication in CV1 cells. Replication kinetics were found to become slower as the inserted gene became longer. The peak titers also became lower, but the reductions were only by several folds at most under single cycle growth conditions (cf. β-gal and control in Fig. 2). As expected, under multiple cycle conditions which involved numerous rounds of replication, more profound differences were seen between the control and each recombinant except SeV/SDF-1α in replication kinetics and the final yield (Fig. 2). Thus, size-dependent replication retardation and titer reduction were both quite clear. These results suggest that the products from inserted genes are so far neutral without giving a particular advantage or disadvantage to the viral replication capability and argues for that the speed of genome replication is inversely correlated simply with the length of inserted genes. A very similar tendency was found with recombinant viruses based upon the V(−) version (Fig. 2). If a pair of recombinant viruses expressing a given gene were compared, the V(−) version always displayed a faster replication than the V(+) version, confirming our previous results [5,6,10].

3.2. Expression of foreign genes from recombinant viruses and its reinforcement with the V(−) version

Fig. 3A illustrates the expression of GFP and β-gal from the respective V(−) recombinant viruses in CV1 cells. Their

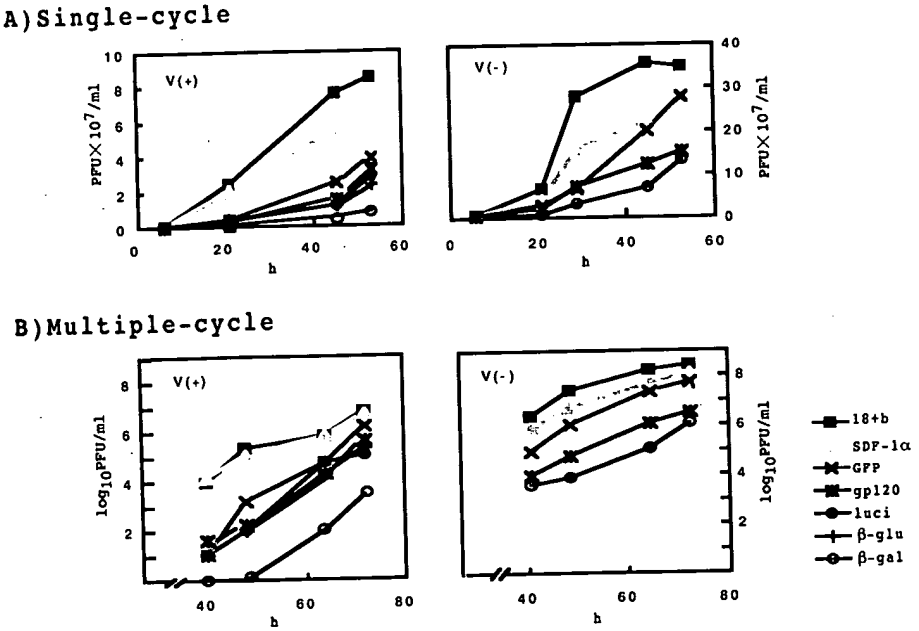

Fig. 2. Replication of various recombinant SeVs in CV1 cells. Growth kinetics of V(+) (A) and V(−) (B) recombinant viruses under the conditions of both single round (multiplicity of infection (moi) is 10 PFU/cell) and multiple rounds (moi 0.01 PFU/cell) of replication are compared.

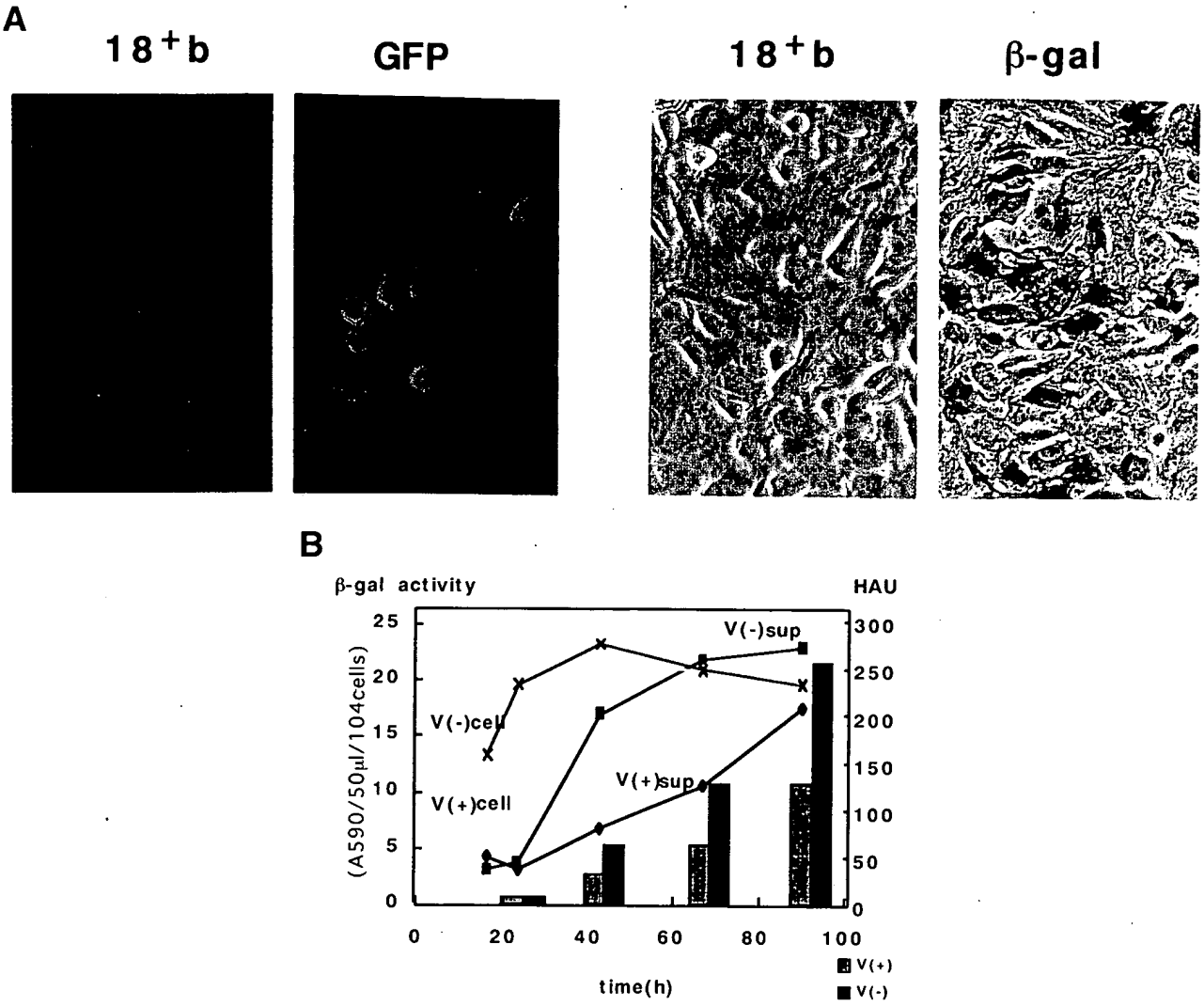

Fig. 3. Expression of foreign genes from recombinant viruses and its reinforcement with the V(−) version. (A) Expression of GFP and β-gal from the respective V(−) recombinant viruses in CV1 cells at 24 h post-infection at a moi of 0.1 PFU/cell. (B) Comparison of intracellular and extracellular β-gal activities expressed from the standard V(+) and V(−) versions at various times post-infection. Virus titers at each time point are shown by HAU.

expression from the V(−) version was already extensive at 24 h post-infection under single cycle growth conditions. Reinforced expression by the V(−) version was confirmed by comparing to intracellular and extracellular β-gal activities expressed from the standard V(+) and V(−) versions (Fig. 3B). Similar vigorous expression from the V(−) SeV in CV1 and other cell lines as well as primary cells of human and avian origins were previously demonstrated for SDF-1α [11], luci [9] and HIV-1 gp120 [10]. These results clearly argue for the utility of SeV, particularly its V(−) version, as a novel expression vector to produce foreign proteins of interest in cultured cells of interest.

3.3. In vivo pathogenicity of recombinant viruses

Fig. 4A shows the replication and pathogenicity of various V(+) recombinant viruses. The virus expressing the shortest gene, SDF-1α, appears to have retained a certain degree of pathogenicity, as body weight gain was greatly disturbed for two of the three mice. Consistent with this, the virus replicated remarkably well and produced high consolidation scores in the lungs. In contrast, the viruses expressing GFP and others longer than the GFP were greatly attenuated, allowing a body weight gain almost comparable to those of the mock-infected mice. This attenuation appeared to be paralleled with lower consolidation scores and less efficient viral replication in the lungs, compared with the control infection. Thus, the extra genes also appeared to affect in vivo viral replication in a length-dependent manner. However, while insertion of a relatively short gene, GFP, resulted in a reduction of the virus titer in the lungs by as much as 100-fold, no further attenuation of replication was seen with longer genes, gp120, luci, β-glu and β-gal. Therefore, in vivo virus growth was not so finely graded by the inserted gene length as in vitro growth. These results suggest the presence of much more complex conditions in vivo where replication would not only be limited by extra gene length but also affected somehow by the biological nature of the gene products and/or differences in host response to the products. The V(−) SeV was previously found to be greatly attenuated in multiplication and pathogenicity in vivo [5,6]. This was confirmed here (Fig. 4B). The V protein appeared to encode a luxury function for the virus to cope with some early host response [5,6]. The V(−) virus is thus

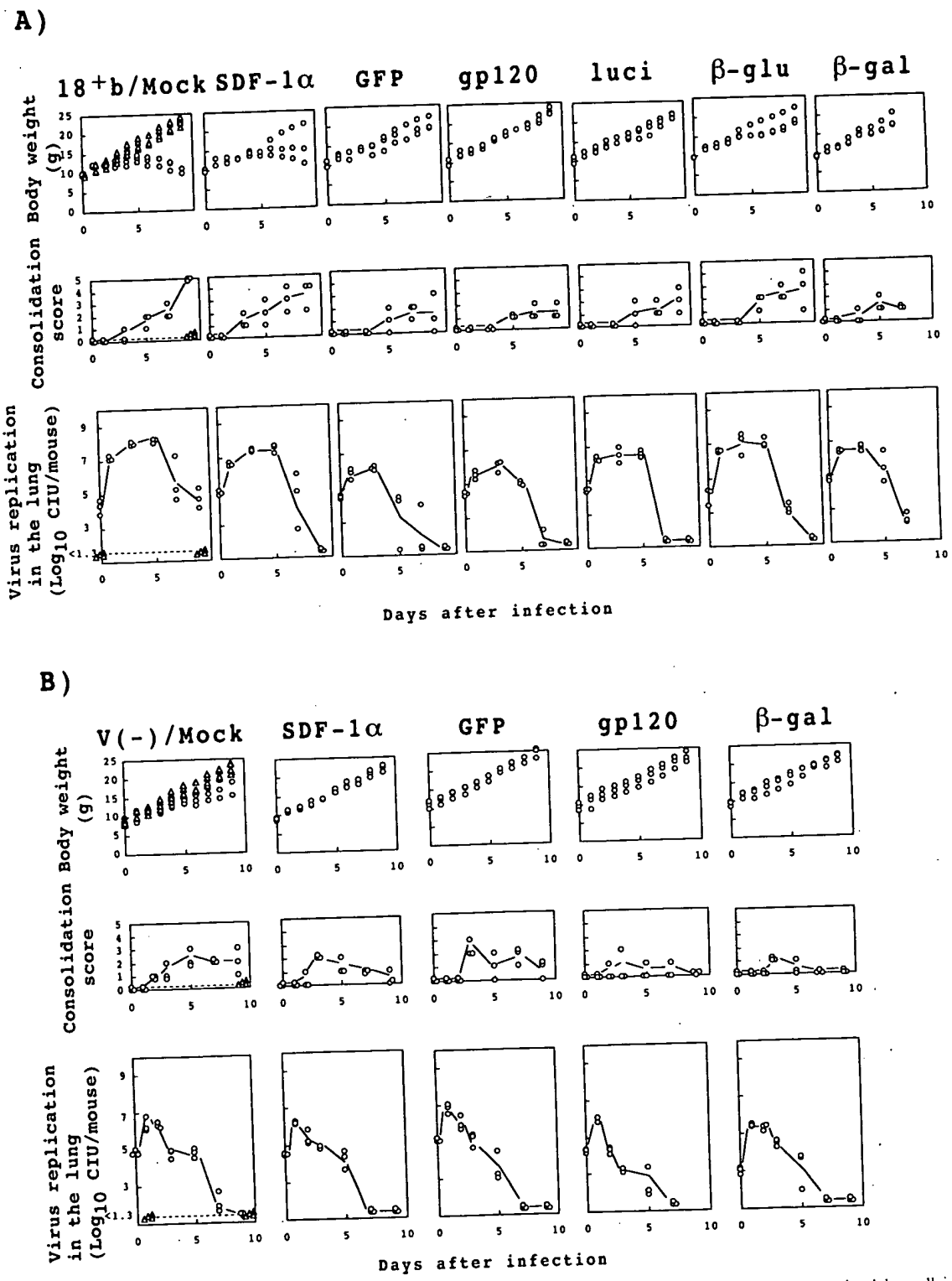

Fig. 4. Replication and pathogenicity of various recombinant viruses in mice. Virus titers in the lungs were determined by cell infectious units per lung and pathogenicity by consolidation scores of the lungs and disturbance of body weight gain as described [5,6]. △, mock-infected.

characterized by an apparently normal rapid replication at the initial day of infection followed by a rapid clearance from the body (Fig. 4B). Its overall in vivo replication pattern was simpler than that of the V(+) counterpart which was featured by maintenance of high virus titers for a long period (Fig. 4A). It may be interesting to note that extra gene length-dependent growth restriction has been seen more clearly in V(−) infections than in V(+) infections (cf. Fig. 4A and B).

4. Discussion

We have previously shown that foreign genes, such as those

encoding SDF-1α, SDF-1β, luci and HIV-1 gp120, can be inserted into and expressed from the SeV genome [9–11]. In this study, we investigated how far SeV can tolerate insertion of foreign genes in terms of their lengths and found that a gene up to 3.2 kb can be readily inserted and expressed. This size accounts for as much as about 1/5 of the original 15 384 bases of the SeV genome. The non-segmented, linear and helical RNP is capable of growing longer and thereby can accommodate such a long extra gene. However, as insertion of a gene longer than 3.2 kb was not attempted, our present study has been unable to define the upper limit.

Viral replication speed must be inversely correlated with the entire genome length. This concept has been used to explain the superiority of the shorter defective interfering viral genome to the full length wild-type genome. However, this has not been illustrated experimentally for any virus. Here, we were able to show for the first time such a genome size-dependent growth retardation because all products from the genes inserted happened, most likely, to be neutral for SeV replication at least in in vitro cells. Probably because of much more complex conditions existing in vivo, the principle could not be very clearly seen in mice but still appeared to be applicable. The fact that recombinant viruses were significantly attenuated as long as the inserted gene was sufficiently long suggests the utility of an even replication competent SeV vector in terms of safety.

Despite the error-prone nature of RNA polymerase in general, maintenance of inserted genes was satisfactory [9]. SeV transcription undergoes polar attenuation toward the 5′ end of thegenome, because the re-initiation at the restart signals of the downstream genes is efficient but not perfect. Consequently, the highest level of foreign gene expression is expected when the gene is placed in the first locus [9]. Furthermore, because of the augmented gene expression of the SeV V(−) mutant (see above), the use of the V(−) version and insertion of a gene upstream of its N ORF guaranteed an extremely high level of expression as shown typically here for β-gal and previously for HIV-1 gp120. The V(−)-based expression level of the latter was the highest currently available in mammalian cells [10]. SeV reaches quite a high copy number in infected cells, is only moderately cytopathic and possesses a broad cellular host range. Hence, replication competent SeV vector, particularly the V(−) version, is extremely useful in producing large quantities of medically important proteins in cells of interest, thereby greatly contributing to functional and structural studies (reviewed in [3]).

Large DNA viruses such as VV have most often been used for production of proteins of interest in mammalian cells. Because of their large genome sizes, e.g. about 190 kb for VV, it has been difficult to create a unique restriction site. Therefore, the step of homologous recombination is necessary for foreign gene insertion. In contrast, because of its short size, a unique *Not*I site has been created in SeV cDNA. This has greatly facilitated insertion of foreign genes in a cassette-like fashion.

The viral vectors most frequently used for gene therapy have derived from retro- and adenoviruses. With the exception of lentiviruses, retroviruses cannot infect non-dividing cells and adenoviruses hardly infect cells of blood origin. Our recombinant SeVs can infect non-dividing cells such as neurons and replicate there and express foreign genes vigorously (in preparation). They also replicate and express foreign genes in the primary human peripheral mononuclear cells and macrophages in culture [10]. SeV replication is independent of nuclear functions and does not have a DNA phase. Thus, it does not transform cells by integrating its genetic information into the cellular genome. Furthermore, homologous recombination has not been observed. Thus, replication incompetent viral constructs grown in complementing cells should be free of a contaminating virus generated by a recombination event. These properties weigh heavily in favor of SeV and related non-segmented negative strand RNA viruses in terms of both utility and safety (reviewed in [3]). Therefore, vectors of the second generation are awaited, which will no longer be replication competent but replication incompetent. They will not encode endogenous envelope proteins but instead incorporate them expressed in *trans* or are pseudotyped with foreign viral envelope proteins.

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ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS

AB Sendai virus (**SeV**) renders cells unresponsive to interferon (IFN)-alpha. To identify viral factors involved in this process, we examined whether recombinant **SeVs**, which could not express V protein, subsets of C proteins (C, C', Y1 and Y2) or any of four C proteins, retained the capability of impeding IFN-a-mediated responses. Among these viruses, only the 4C knockout virus completely lost the ability to suppress the induction of IFN-alpha-stimulated gene products and the subsequent establishment of an anti-viral state. These findings reveal crucial roles of the **SeV** C proteins in blocking IFN-alpha-mediated responses. (C) 1999 Federation of European Biochemical Societies.

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Knockout of the Sendai virus C gene eliminates the viral ability to prevent the interferon-α/β-mediated responses

Bin Gotoh[a,*], Kenji Takeuchi[a], Takayuki Komatsu[a], Junko Yokoo[b], Yoshinobu Kimura[a], Atsushi Kurotani[d], Atsushi Kato[c,d], Yoshiyuki Nagai[d,e]

[a] *Department of Microbiology, Fukui Medical University, Shimoaizuki 23-3, Matsuoka-cho, Yoshida-gun, Fukui 910-1193, Japan*
[b] *Radioisotope Research Institute, Fukui Medical University, Shimoaizuki 23-3, Matsuoka-cho, Yoshida-gun, Fukui 910-1193, Japan*
[c] *Department of Viral Diseases and Vaccine Control, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-murayama, Tokyo 208-0011, Japan*
[d] *Department of Viral Infection, Institute of Medical Science, University of Tokyo, Shirokanedai 4-6-1, Minato-ku, Tokyo 108-8639, Japan*
[e] *AIDS-Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan*

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Abstract **Sendai virus (SeV) renders cells unresponsive to interferon (IFN)-α. To identify viral factors involved in this process, we examined whether recombinant SeVs, which could not express V protein, subsets of C proteins (C, C′, Y1 and Y2) or any of four C proteins, retained the capability of impeding IFN-α-mediated responses. Among these viruses, only the 4C knockout virus completely lost the ability to suppress the induction of IFN-α-stimulated gene products and the subsequent establishment of an anti-viral state. These findings reveal crucial roles of the SeV C proteins in blocking IFN-α-mediated responses.**

Key words: Sendai virus; C protein; Interferon; Interferon-stimulated gene; Stat1; p48

1. Introduction

Interferons (IFNs) play important roles in early steps of host defense mechanisms and exert their biological effects through induction of IFN-stimulated gene (ISG) products including anti-viral proteins such as double-stranded RNA (dsRNA) dependent protein kinase (PKR) and 2′,5′-oligoadenylate synthetase (2-5AS) [1]. Binding of IFN-α/β to the cell surface receptors initiates activation of the receptor-associated tyrosine kinases, Jak1 and Tyk2. These activated kinases phosphorylate specific tyrosine residues of signal transducers and activators of transcription, Stat1 and Stat2, which then combine to p48 and migrate to the nucleus to function as active ISG factor 3 (ISGF3) [2,3]. ISGF3 binds to the IFN-stimulated response element (ISRE) in target genes and activates transcription of ISGs [4].

A variety of viruses have developed sophisticated strategies to counteract IFN-mediated induction of anti-viral states [5]. For example, adenovirus [6] and Epstein-Barr virus [7,8] prevent activation of PKR by producing abundant small RNA molecules that can bind to the dsRNA-binding site of PKR, whereas vaccinia virus and reovirus express dsRNA-binding proteins to prevent dsRNA from activating PKR [9–12]. In contrast to mechanisms directed to the anti-viral proteins, several viruses developed more ingenious strategies in which the IFN signal transduction is prevented [13]. The strategies could theoretically lead to suppression of all (more than 50) ISG products. Poxviruses including vaccinia virus and myxoma virus encode soluble IFN receptor homologues [14–18]. Adenovirus inhibits formation of ISGF3 by decreasing p48 [19,20], while human cytomegalovirus decreases Jak1 as well as p48, two essential components of the IFN-α signaling pathway [21]. Little is known, however, about whether these strategies are common among viruses.

We have recently found that Sendai virus (SeV), a prototype paramyxovirus, also has the ability to suppress the anti-viral action of IFN-α [22]. SeV is likely to be one of the viruses evolving the ingenious strategies, since the activation of an IFN-α/β-responsive promoter was inhibited in the SeV-infected cells [23]. Thus, we expected that detailed analysis of the suppression mechanism of SeV would elucidate a novel viral strategy directed to the IFN signal transduction. In this study, we especially focussed on viral factors responsible for the suppression mechanism. This mechanism was very unique and required neither viral genome replication nor secondary transcription [22]. In fact, Sendai virions inactivated by short-term UV-irradiation retained the ability to interfere with the IFN-mediated induction of an anti-viral state [22]. Thus, we speculated that responsible viral factors were encoded by the 3′ upstream region of the SeV genome such as the N or P/C gene (Fig. 1A). On the other hand, recent studies using gene knockout SeVs, which were created by technology of the reverse genetics [24,25], have demonstrated that the C and V proteins play crucial roles in in vivo growth and viral pathogenicity [26–30]. These findings prompted us to examine whether the C and V knockout SeVs retained the ability to impede IFN-mediated responses.

In addition to the previously generated V(−), C/C′(−) and 4C(−) viruses, which were unable to express the V, C plus C′ and any of four C (C, C′, Y1 and Y2) proteins, respectively, we used newly created recombinant C′(−) virus, which does not express C′ protein, in this study. We show here that among these viruses, only the 4C(−) virus completely loses the ability to prevent induction of ISG products as well as the establishment of an anti-viral state.

2. Materials and methods

2.1. Generation of recombinant SeV

Generation of 4C(−), C/C′(−) and V(−) viruses was described previously [27,29]. The C′(−) virus was newly generated essentially

*Corresponding author. Fax: (81) (776) 61 8104.
E-mail: bin@fmsrsa.fukui-med.ac.jp

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as previously described [25,29]. The initiation codon for C′ was disrupted by site-directed mutagenesis (Fig. 1B). A pair of complementary primers (CP1: ^{1830}5′-CGAAGCCGcGGCTGTCTCGAC-3′^{1809} and CP2: ^{1809}5′-GTCGAGACAGCCgCGGCTTCG-3′^{1830}), which contain a mutation (shown by lower-case letters) in the initiation codon for C′, and outer primers (OP1: ^{61}5′-CAAAGTATCCACCCTGAGGAGCAGGTTCCAGACCCTTTGCTTTGC-3′^{105} and OP2: ^{2468}5′-TTACTCTTGCACTATGTG-3′^{2451}) were used for generation of the mutated DNA fragment by two-step PCR-based overlap primer extension [31]. Nucleotides of primers were numbered according to Shioda et al. [32]. The mutated DNA fragment was digested with *Sph*I and *Sal*I and inserted to the corresponding position in pSeV(+), which can generate a full length copy of parental SeV positive sense antigenome, to create the mutant pSeV(+)/C′(−). Using the pSeV(+)/C′(−), the recombinant C′(−) virus was recovered as previously described [25,29]. Mutagenesis in the P/C gene of 4C(−), C/C′(−), C′(−) and V(−) viruses is summarized in Fig. 1B,C.

2.2. Cell cultures and virus propagation

HeLa and Vero cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% bovine serum, 10% tryptose phosphate broth and 60 μg/ml kanamycin. Vesicular stomatitis virus (VSV) strain New Jersey was propagated in BHK cells. Recombinant SeV stocks, cDNA-derived Z strain as a wild-type (WT), 4C(−), C/C′(−), C′(−) and V(−) viruses (Fig. 1B,C) [27,29], were prepared by harvesting culture supernatant of virus-infected Vero cells incubated for 3–7 days at 32°C in serum-free MEM in the presence of 1 μg/ml of trypsin. Hemagglutinin titers were measured as previously described [33].

2.3. IFN and antibodies

Recombinant human IFN-α-2a was purchased from Takeda Chemical Industries (Osaka, Japan). Anti-PKR rabbit polyclonal antibody (SC-707), anti-p48 rabbit polyclonal antibody (SC-496), anti-Stat1 mouse monoclonal antibody (SC-464) and anti-Stat2 rabbit polyclonal antibody (SC-476) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse neutralizing monoclonal antibody against human IFN-α (MMHA-2) and rabbit polyclonal antibody against human IFN-β were purchased from Cosmo Bio (Tokyo, Japan).

2.4. Assay of virus titers

VSV was titrated by the plaque assay on BHK cells. It was critical for this study to determine conditions under which all cells were actually infected with each recombinant SeV. Since it was difficult to determine exact titers of 4C(−) virus by the plaque assay, an alternative titration method, hemadsorption test, was employed for SeV. Cells in 24 well plates were infected with sequentially diluted SeV stock. At 20 h post-infection (pi), 1% chicken erythrocytes in phosphate-buffered saline were added to the cells. After incubation at 4°C for 30 min, cells were extensively washed and then fixed with chilled methanol. The maximum dilution of virus stocks, at which 100% of cells adsorbed erythrocytes, was determined. For all the experiments in this study, cells in 24 well plates were infected with SeV at around a 3-fold higher concentration relative to the maximum dilution of virus stocks or infected with VSV at a multiplicity of infection of 10 plaque forming units/cell.

2.5. Immunofluorescent staining

Cells propagated on round cover glasses (11 mm diameter) were fixed with chilled methanol and then stained by the double immunofluorescent method with anti-SeV rabbit serum and anti-VSV mouse serum. Rhodamine-labelled anti-rabbit IgG goat serum (Tago, Burlingame) and FITC-labelled anti-mouse IgG goat serum (Tago, Burlingame) were used as the second antibodies, respectively.

2.6. Western blotting

All steps were carried out at 0–4°C. Cells in 24 well plates were lysed in 25 μl of extraction buffer (50 mM HEPES, pH 7.6, 300 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and 2 μg/ml pepstatin) according to Lee et al. [34]. Each lysate (40 or 1 μg protein) was electrophoresed in 7.5 or 10% sodium dodecyl sulfate-polyacrylamide gels [35] for analysis of ISG products (Stat1, Stat2, p48 and PKR) or virus proteins, respectively. The proteins in the gels were electrotransferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA, USA) and probed with the specific antibodies. As the second antibodies, horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies (Amersham Pharmacia Biotech, UK) were used. The proteins were detected using ECL detection reagent (Amersham Pharmacia Biotech, UK). The protein concentration was determined by the BCA protein assay kit (Pierce, Rockford, USA).

3. Results

The genome construct of SeV is shown in Fig. 1A. SeV has a non-segmented negative strand RNA genome of 15384 nucleotides, which encodes the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN) and large protein (L) as structural proteins. Only the P/C gene exceptionally encodes multiple species of proteins, the V, W, X, C′, C, Y1 and Y2 proteins besides the P protein (Fig. 1A) [36,37]. The V and W mRNAs are generated by pseudotemplated addition of one and two G residue(s) at a specific P/C gene region (1051–1053) (Fig. 1A,C), termed RNA editing, while the unedited exact copy of the P/C gene encodes the P protein. Therefore, the N-terminal amino acid sequence is shared with the P, V and W proteins (Fig. 1A). On the other hand, the C proteins, a nested set of four proteins (C′, C, Y1 and Y2),

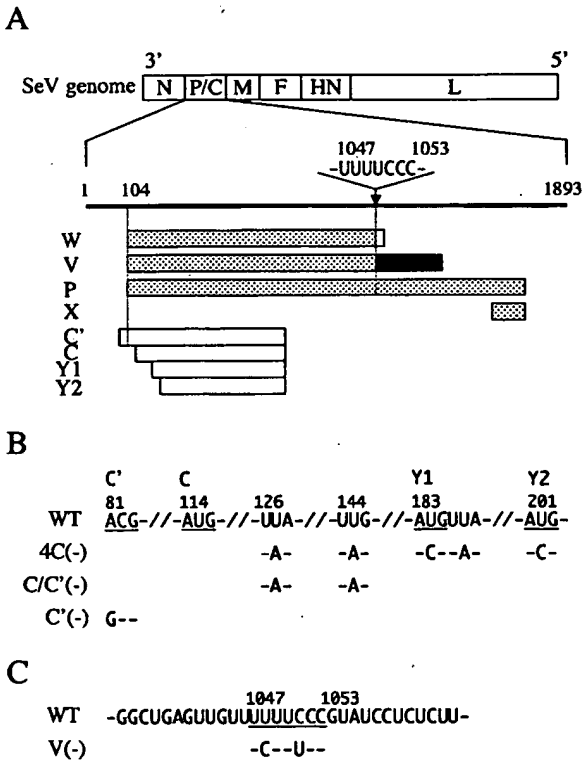

Fig. 1. Mutagenesis in the P/C gene for generation of 4C(−), C/C′(−), C′(−) and V(−) viruses. (A) The genome construct of SeV and protein species encoded by the P/C gene. The P/C gene encodes multiple proteins, V, W, X, C′, C, Y1 and Y2. The P ORF is shown as a dotted box, the V ORF in the −1 frame as a black box and the C ORF (+1) as an open box. (B) Sequence around the multiple initiation codons (underlined) in P mRNA and mutagenesis for generation of 4C(−), C/C′(−) and C′(−) viruses. Mutations were introduced to disrupt the initiation codons (114–116, 183–185 and 201–203) or to create the termination codons (126–128, 144–146 and 185–187). (C) Sequence around the RNA editing signal (underlined) in the genome and mutagenesis for generation of V(−) virus.

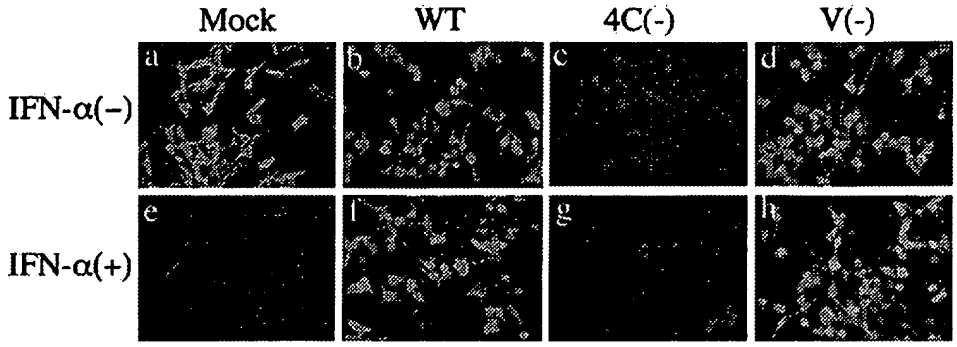

Fig. 2. Ability of WT, 4C(−) virus and V(−) viruses to rescue replication of VSV from the anti-viral action of IFN-α. HeLa cells were mock-infected (Mock) or infected with each recombinant SeV. One h after infection, the media were replaced with fresh media containing IFN-α (1000 IU/ml) or no IFN-α. Following incubation for 15 h, cells were superinfected with VSV. After further incubation for 6 h, cells were fixed with chilled methanol and stained by the double immunofluorescent method as described in Section 2. Only VSV fluorescence was shown.

initiated at different initiation codons, are encoded by an open reading frame (ORF) overlapping the P ORF in the +1 frame (Fig. 1A). The V(−), C′(−), C/C′(−) and 4C(−) viruses, which are unable to express the V, C′, C plus C′ and four C proteins, respectively, were generated as described in Section 2.

3.1. Loss of the ability of 4C(−) virus to impede the anti-viral action of IFN-α/β

VSV, one of the highly IFN-α/β-sensitive viruses, is used to assess whether an anti-viral state is established by IFN-α/β or not. First, we examined the ability of V(−) and 4C(−) viruses to rescue of VSV replication. HeLa cells were mock-infected or infected with WT, 4C(−) or V(−) virus and then treated with IFN-α (1000 IU/ml) at 1 h pi. Sixteen h after SeV infection, the cells were superinfected with VSV and further incubated for 6 h. Then, the cells were fixed and stained doubly by anti-VSV and anti-SeV antibodies. As shown in Fig. 2, VSV antigens were detected in most cells pre-infected with WT virus despite treatment with IFN-α (Fig. 2b,f) as described previously [22]. On the other hand, most of the 4C(−) virus-infected cells exhibited very little or no VSV fluorescence irrespective of treatment with exogenous IFN-α (Fig. 2c,g). In cells infected with V(−) virus (Fig. 2d,h), results were similar to those obtained in the WT virus-infected cells (Fig. 2b,f), although the ratio of cells with apparent VSV fluorescence to those with little fluorescence seemed to be lower than that in the WT virus infection on the whole. The absence of VSV antigens in the 4C(−) virus-infected cells was not due to failure of SeV infection since the double staining method confirmed that all cells were certainly infected with 4C(−) virus (data not shown). These results showed that V(−) virus retained the ability to block the IFN-mediated induction of an anti-viral state comparable to that of WT virus (Fig. 2b,d,f,h), suggesting that the V protein is not involved in this anti-IFN mechanism. On the other hand, 4C(−) virus appeared to lose the anti-IFN ability completely. It was, however, not clear why 4C(−) virus-infected cells did not allow for VSV replication even in the absence of IFN-α. We speculated that autocrine IFN-α/β induced by the SeV infection established an anti-viral state in the 4C(−) virus-infected cells because of the loss of the anti-IFN ability of 4C(−) virus. In fact, it is known that both IFN-α and IFN-β mRNAs are induced in HeLa cells in response to virus infection [38].

To check this, we examined effects of anti-IFN-α/β antibodies added to the medium on rescue of VSV replication. Fig. 3A showed Western blotting analysis of VSV proteins in infected cells in the presence or absence of anti-IFN-α/β anti-

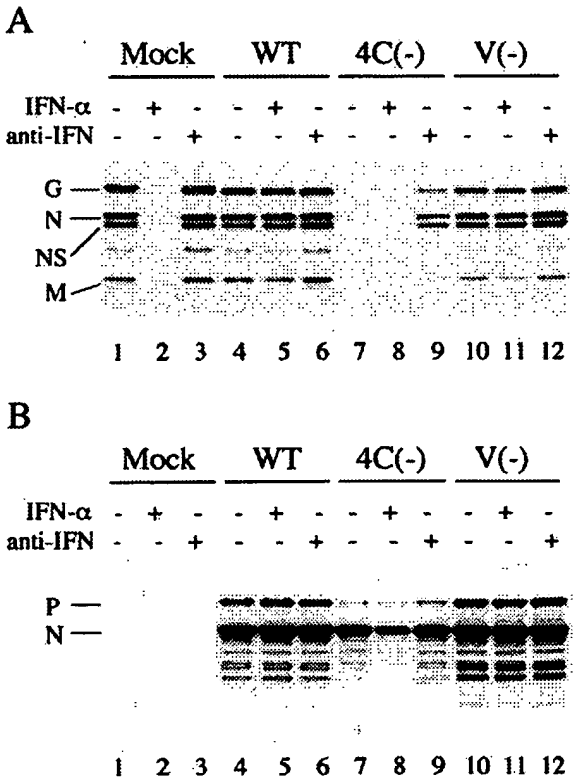

Fig. 3. Ability of WT, 4C(−) and V(−) viruses to suppress the anti-viral action of exogenous IFN-α and autocrine IFN-α/β. HeLa cells were mock-infected (Mock) or infected with each SeV. Two h after infection, the culture media were replaced with fresh media containing IFN-α (1000 IU/ml) (lanes 2, 5, 8 and 11) or no IFN-α (lanes 1, 4, 7 and 10). After incubation for 18 h, the cells were superinfected with VSV. Then, the cells were harvested at 6 h pi with VSV. The total cell extracts were subjected to Western blotting analysis with anti-VSV (A) or anti-SeV (B) serum. When cells were treated with anti-IFN-α/β antibodies (lanes 3, 6, 9 and 12), the culture media contained both neutralizing mouse monoclonal anti-IFN-α (final concentration 40 μg/ml) and rabbit polyclonal anti-IFN-β (final concentration 3000 U/ml) throughout the experiments.

bodies or exogenous IFN-α. Exogenously added IFN-α completely inhibited production of all viral components of VSV in HeLa cells (Fig. 3A, lane 2), while infection with WT or V(−) virus allowed for VSV protein synthesis (Fig. 3A, lanes 5 and 11). In the 4C(−) virus-infected cells, however, no accumulation of VSV proteins was observed irrespective of IFN-α treatment (Fig. 3A, lanes 7 and 8). These results were in good agreement with those in the immunofluorescent experiment described above.

Anti-IFN-α/β antibodies treatment did not affect notably VSV protein synthesis in WT or V(−) virus-infected cells (Fig. 3A, lanes 6 and 12). In contrast, the accumulation of VSV antigens was dramatically enhanced in the 4C(−)-infected cells (Fig. 3A, lane 9), indicating that autocrine IFN-α/β at least in part contributed to suppression of VSV protein synthesis in the 4C(−) virus-infected cells without exogenous IFN-α. We also examined SeV protein synthesis in the infected cells. Accumulation of the 4C(−) virus proteins was found to be less than that of WT or V(−) virus proteins (Fig. 3B, lanes 4, 7 and 10). Neither anti-IFN-α/β antibodies nor exogenous IFN-α affected WT and V(−) virus protein syntheses (Fig. 3B, lanes 6 and 12), while the 4C(−) virus protein synthesis was found to be suppressed or recovered to some extent by IFN-α treatment or by anti-IFN-α/β antibodies treatment, respectively (Fig. 3B, lanes 8 and 9). These results demonstrated that SeV protein synthesis was actually influenced by autocrine IFN-α/β in the absence of the SeV C proteins.

3.2. *Complete loss of the ability of 4C(−) virus to suppress induction of ISG products*

As described above, an anti-viral state was induced in the 4C(−) virus-infected cells, but not in the WT and V(−) virus-infected cells. To see this difference at a level of host cell gene expression, we examined the ability of each virus to impede induction of ISG products. Stat1, Stat2 and p48, components of ISGF3, were chosen for the ISG products [39] besides PKR. HeLa cells were mock-infected or infected with each SeV and treated or untreated with IFN-α at 2 h pi. Cells were harvested 18 h after IFN-α treatment. Fig. 4 showed that IFN-α treatment remarkably increased Stat1 (Stat1α and Stat1β) and p48, in the 4C(−) virus-infected cells (Fig. 4, lane 11) comparable with those in mock-infected cells (Fig. 4, lane 3), whereas in the WT or V(−) virus-infected cells, induction of Stat1 and p48 by autocrine IFN-α/β or exogenous IFN-α was strikingly suppressed (Fig. 4, lanes 6, 7, 14 and 15). As expected, even in the absence of exogenous IFN-α, Stat1 and p48 were markedly induced in the 4C(−) virus-infected cells (Fig. 4, lane 10). On the other hand, anti-IFN-α/β antibodies suppressed increase of these ISG products in the 4C(−)-infected cells (Fig. 4, lane 12), confirming that autocrine IFN-α/β actually acted to establish the anti-viral state on the 4C(−) virus-infected cells. Similar results were obtained in the induction of Stat2 and PKR (data not shown). These results, consistent with those observed in Fig. 3, suggested that the 4C(−) virus allowed for the establishment of an anti-viral state in the infected cells by the complete loss of the ability to prevent induction of ISG products.

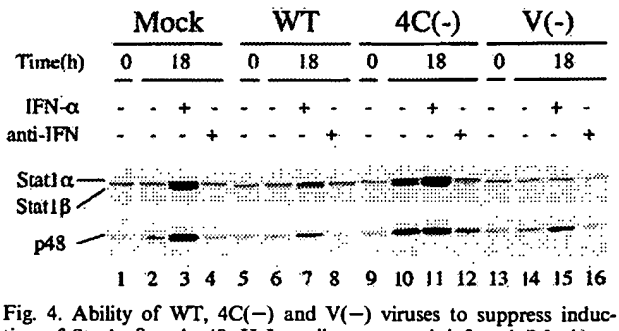

Fig. 4. Ability of WT, 4C(−) and V(−) viruses to suppress induction of Stat1α,β and p48. HeLa cells were mock-infected (Mock) or infected with each SeV. The culture media were replaced at 2 h pi with fresh media containing IFN-α (1000 IU/ml) (lanes 3, 7, 11 and 15) or no IFN-α (lanes 1, 2, 5, 6, 9, 10, 13 and 14). The cells were further incubated for 18 h until cell harvesting. When cells were treated with anti-IFN-α/β antibodies, the treatment conditions were the same as those in Fig. 3. The total cell lysates were analyzed by Western blotting with anti-Stat1 and anti-p48 antibodies.

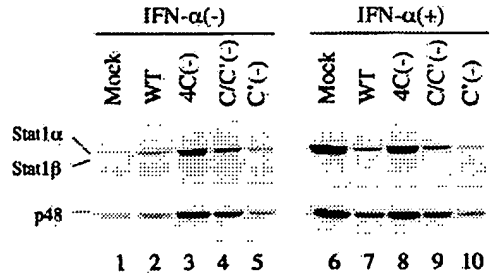

Fig. 5. Ability of C/C′(−) and C′(−) viruses to suppress induction of Stat1 and p48. HeLa cells were mock-infected (Mock) or infected with each virus. The media were replaced at 2 h pi with fresh media containing IFN-α (1000 IU/ml) (lanes 6–10) or no IFN-α (lanes 1–5). The cells were harvested after a further 18 h incubation. The total cell lysates were analyzed by Western blotting with anti-Stat1 and anti-p48 antibodies.

3.3. *Ability of C/C′(−) and C′(−) viruses to suppress induction of ISG products*

The ability of C/C′(−) and C′(−) viruses to suppress induction of ISG products was also examined. In the C/C′(−) virus-infected cells, Stat1 and p48 were induced to some extent probably by autocrine IFN-α/β in the absence of exogenous IFN-α (Fig. 5 lane 4), but the level of the induction was inferior to that in the 4C(−) virus-infected cells (Fig. 5 lane 3). Induction of Stat1 and p48 was strongly suppressed in the C′(−) virus-infected cells (Fig. 5, lane 5). When cells were treated with exogenous IFN-α, the difference between the 4C(−) and the other viruses became more evident. Induction of Stat1 and p48 in the C/C′(−) virus-infected cells was found to be suppressed considerably compared with that in the 4C(−) virus-infected cells. The suppression by C′(−) virus appeared to be stronger than that of WT virus. Similar results were obtained in the induction of Stat2 and PKR (data not shown). The Y1 and Y2 proteins were overexpressed in HeLa cells infected with C/C′(−) virus (data not shown) as previously described [29]. Therefore, overexpressed Y1 and Y2 proteins largely compensated for the loss of functions of the C and C′ proteins and could contribute to suppression of induction of the ISG products to some extent.

4. Discussion

The present study demonstrated that both WT and V(−)

viruses blocked the induction of ISG products (Stat1, p48, Stat2 and PKR) and consequently prevented the establishment of an anti-viral state. Didcock et al. reported that transfection of a plasmid carrying a synthetic promoter containing multimers of the well-defined ISRE linked to the luciferase reporter gene did not respond to IFN-α/β in cells infected with SeV [23]. They further showed that induction of the IFN-responsive 6-16 gene was inhibited by SeV infection. Together with their results, our findings support the idea that SeV interferes with the IFN-α/β signal transduction.

In contrast to WT and V(−) viruses, the 4C(−) virus completely lost the ability to suppress the IFN-mediated responses, demonstrating that the C proteins play crucial roles in preventing IFN-mediated induction of an anti-viral state. It is unlikely that a low accumulation of SeV proteins in infected cells (Fig. 3B, lane7) is responsible for loss of the anti-IFN ability of 4C(−) virus, because even replication-incompetent SeV can suppress the IFN-α/β-mediated anti-viral responses [22]. Rather, previous double immunostaining experiments showed that detectable levels of viral proteins were not required for the anti-IFN ability [22]. Recently, Garcin et al. have reached essentially the same conclusion using mutant viruses containing a single amino acid substitution in the C protein and similar recombinant viruses which do not express subsets of the C proteins [40]. They concluded that the AUG^{114}-initiated C protein prevents the establishment of an anti-viral state, from the results showing that not only the double mutant corresponding to the C/C′(−) virus but also the C(−) mutant allowed for IFN-α/β-mediated induction of Stat1 and lost the ability to rescue VSV. We, however, found that C/C′(−) virus retained the considerable ability to suppress the ISG products, although the suppression is slightly weak compared with WT and C′(−) viruses (Fig. 5). Therefore, our results suggest that Y1 and Y2 proteins can also play a critical role in prevention of the IFN-mediated responses. Although the reasons for the discrepancies between our and their results are unknown, they might be due to a difference in cell lines used or experimental conditions.

Growth of 4C(−) virus in ovo was very poor and was reduced by several logs, compared with that of WT virus [29]. Even CC′(−) virus was almost totally incapable of replicating in the mouse lung [29]. Therefore, 4C(−) virus is likely to be more avirulent than CC′(−) virus. This possible attenuation as well as poor growth of 4C(−) virus in ovo is explainable in part by loss of the ability of 4C(−) virus to prevent autocrine IFN-α/β from inducing an anti-viral state, as shown in our in vitro study presented here. Although SeV protein synthesis was actually suppressed by autocrine IFN-α/β in the 4C(−) virus-infected HeLa cells (Fig. 3B), its extent was not so striking when compared with the inhibition of VSV protein synthesis (Fig. 3A). This difference is probably due to the timing of the action of IFN-α/β. In the experiments shown in Fig. 3A, VSV infection followed establishment of the anti-viral state (Fig. 4), while SeV infection preceded it. Thus, the timing of virus infection to IFN treatment is an important factor for blocking IFN-mediated responses. We confirmed that SeV infection could not prevent IFN-mediated responses in cells pre-treated with IFN-α as reported by Didcock et al. [23]. On the other hand, it is well known that SeV is a good inducer of IFN-α/β. Therefore, it remains to be elucidated why WT virus can spread to neighboring cells in which an anti-viral state has already been established by the action of paracrine IFN-α/β. Why has SeV evolved the anti-IFN strategy in contrast to VSV? We previously reported that VSV protein synthesis was not inhibited at all by IFN-β treatment, if IFN-β was added to cells concomitantly with VSV infection [41]. Therefore, it is unlikely that VSV growth is affected by autocrine IFN-α/β. VSV, due to its rapid growth, may not need an anti-IFN strategy like SeV. Thus, we speculate that SeV must have evolved the anti-IFN strategy in compensation for its slower growth.

We observed acceleration of cytopathic effects in the 4C(−) virus-infected HeLa cells (IFN-α/β producing cells) compared with those in the WT virus-infected cells (unpublished results), although we did not determine whether the cytopathic effects were due to apoptosis. Therefore, the anti-IFN mechanisms may be linked to induction of apoptosis as speculated by Garcin et al. [40], since activation of PKR or 2-5AS induces apoptosis [42–45]. If the ability of SeV to inhibit IFN-α/β-mediated responses is also responsible for delay in deterioration of cell functions, it will be of advantage to the virus growth.

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Large quantity production with extreme convenience of human SDF-1.alpha. and SDF-1.beta. by a **Sendai virus vector**
Moriya, Chikaya; Shioda, Tatsuo; Tashiro, Kei; Nagasawa, Takashi; Ikegawa, Masaya; Ohnishi, Yukano; Kato, Atsushi; Hu, Huiling; Xin, Xiaomi; Hasan, Mohammad K.; Maekawa, Midori; Takebe, Yutaka; Sakai, Yuko; Honjo, Tasuku; **Nagai, Yoshiyuki**
RPORATE SOURCE: Minato-ku, 4-6-1 Shiroganedai, Institute of Medical Science, Department of Viral Infection, University of Tokyo, Tokyo, 108, Japan
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AB We describe a robust expression of human stromal cell-derived factor-1.alpha. (SDF-1.alpha.) and SDF-1.beta., the members of CXC-chemokine family, with a novel **vector** system based upon **Sendai virus**, a non-segmented neg. strand **RNA** virus. Recombinant SDF-1.alpha. and SDF-1.beta. were detected as a major protein species in culture supernatants, reached as high as 10 .mu.g/mL. This remarkable enrichment of the products allowed us to use even the crude supernatants as the source for biol. and antiviral assays without further concn. nor purifn. and will thus greatly facilitate to screen their genetically engineered derivs.

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Large quantity production with extreme convenience of human SDF-1α and SDF-1β by a Sendai virus vector

Chikaya Moriya[a], Tatsuo Shioda[a,b], Kei Tashiro[c], Takashi Nagasawa[d], Masaya Ikegawa[f], Yukano Ohnishi[a], Atsushi Kato[a], Huiling Hu[a], Xiaomi Xin[a], Mohammad K. Hasan[a], Midori Maekawa[g], Yutaka Takebe[h], Yuko Sakai[a], Tasuku Honjo[e], Yoshiyuki Nagai[a,*]

[a]*Department of Viral Infection, Institute of Medical Science, University of Tokyo, 4-6-1 Shirogandai, Minato-ku, Tokyo 108, Japan*
[b]*Department of Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo, Japan*
[c]*Center for Molecular Biology and Genetics, Kyoto University, Kyoto, Japan*
[d]*Department of Immunology, Research Institute, Osaka Medical Center for Maternal and Child Health, Osaka, Japan*
[e]*Department of Medical Chemistry, Kyoto University, Kyoto, Japan*
[f]*Department of Social Medicine, Kyoto University, Kyoto, Japan*
[g]*Department of Pharmacology, Kyoto University, Kyoto, Japan*
[h]*Department of Molecular Virology and Epidemiology, AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan*

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Abstract **We describe a robust expression of human stromal cell-derived factor-1α (SDF-1α) and SDF-1β, the members of CXC-chemokine family, with a novel vector system based upon Sendai virus, a non-segmented negative strand RNA virus. Recombinant SDF-1α and SDF-1β were detected as a major protein species in culture supernatants, reached as high as 10 μg/ml. This remarkable enrichment of the products allowed us to use even the crude supernatants as the source for biological and antiviral assays without further concentration nor purification and will thus greatly facilitate to screen their genetically engineered derivatives.**

Key words: HIV-1; Chemokine; Stromal cell-derived factor 1; Sendai virus; Virus vector; Mammalian expression system

1. Introduction

Stromal cell-derived factor (SDF-1), also named pre-B cell growth-stimulating factor (PBSF), is a member of the CXC-chemokine family. SDF-1 was initially defined as a bone marrow stromal cell-derived soluble factor [1], but is subsequently found to be considerably multi-functional as it is involved in B lymphopoiesis, bone marrow hematopoiesis and cardiac ventricular septal formation [2,3]. Human and murine SDF-1 is known to arise in two forms, SDF-1α and SDF-1β, by differential splicing from a single gene. They differ in four carboxy-terminal amino acid residues which are present in SDF-1β and absent in SDF-1α [1,4]. Chemokines constitute a large family of small chemotactic cytokines of 60–80 amino acid residues. The members of this family have four conserved cysteine residues which form two intra-molecular disulfide bridges [5]. There are two subfamilies of chemokines, CC-chemokines and CXC-chemokines, which differ in the spacing of the first two cysteine residues. The CC-chemokine subfamily includes macrophage inflammatory peptide-1α and -1β (MIP-1α and MIP-1β), regulated on activation normal T cell expressed and secreted (RANTES) and monocyte chemoattractant protein-1 (MCP-1). The CXC-chemokine subfamily includes interleukin-8 (IL-8), platelet factor-4 (PF-4), in addition to SDF-1. Of these chemokines, SDF-1 appears to be most efficacious as a chemoattractant on resting T lymphocytes and monocytes [6].

Recently, both CC- and CXC-chemokines are attracting the keenest attention, because their receptors were proved to be used by human immunodeficiency virus type 1 (HIV-1) as a co-receptor for its entry into the CD4+ cells [7–13], and because they competitively block this virus-cell interaction [14–18]. Co-receptor usage and hence inhibition of infection by chemokines is strain specific. For instance, macrophage tropic/non-syncytium inducing strains need CCR-5 for their entry into target cells [7,9–12] and their infection can be blocked by the corresponding ligands, MIP-1α, MIP-1β, and RANTES [14,16,18]. On the other hand, CXCR-4 serves as a co-receptor for T cell line tropic/syncytium inducing strains [8,13], and its ligand SDF-1 can block the infection [15,17]. These discoveries have not only greatly facilitated our understanding of HIV replication and pathogenesis but also opened a novel possibility to treat HIV infection with chemokines or chemokine derivatives.

MIP-1α, MIP-1β and RANTES, produced in *E. coli*, have become commercially available and now widely used to get more information on the mechanism of co-receptor mediated HIV entry and its block by chemokines [7,18–21]. However, *E. coli*-based production generally requires extensive, multi-step purification of the product before use, and therefore is not always feasible for testing many different, genetically engineered derivatives. Extensive aggregation is often inevitable particularly for such basic polypeptides as chemokines. Thus, chemical synthesis of the original and modified versions has been adopted. This approach is not only laborious, including careful refolding, but also expensive. Purification of recombinant chemokines produced in mammalian and other higher vertebrate cells by recombinant viruses has not been reported.

We recently established a system to recover infectious Sendai virus (SeV), a non-segmented negative strand RNA virus in the family Paramyxoviridae, entirely from cDNA [22], and further succeeded in applying the technology to insert a foreign gene of interest to SeV genome and express the gene in extremely large quantities from an infectious recombinant SeV [23,24]. Here, we made attempts to create recombinant SeV expressing SDF-1α and SDF-1β to know whether or not this

*Corresponding author. Fax: +81 (3) 5449-5409.
E-mail: ynagai@ims.u-tokyo.ac.jp

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system can be applied for large quantity production of biologically active cytokines which can be used with minimal purification procedures. The SDF-1α and SDF-1β expressed from recombinant viruses consistently reached in amounts as high as 10 mg or more per liter of tissue culture medium. This high level of accumulation of the products has allowed not only the use of crude tissue culture medium as probes for chemotaxis and antiviral assays but also their purification by a single-step column chromatography. Thus, our system represents a novel and useful option for providing SDF-1α and SDF-1β and probably other chemokines and cytokines.

2. Materials and methods

2.1. Viruses and cells

HIV-1 strains NL43 [25], SF33 [26], and TK11 [27] and SIV mac strain 239 [28] were grown in MT4 T cell line. HIV-1 strain SF162 [29], primary isolates #12, #15 and #37 [30,31] were propagated in phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMC). CV1 cells were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). MT4 cells were grown in RPMI-1640 supplemented with 10% FBS. Primary chicken embryo fibroblasts (CEF) were prepared as described previously [32], and maintained in MEM supplemented with 10% FBS. After virus infection, CEF were maintained in MEM without serum. PBMC from healthy seronegative donors were prepared and grown as described previously [30].

2.2. Generation of recombinant Sendai viruses

Human SDF-1α or SDF-1β cDNAs were inserted just upstream of the open reading frame of the 3' proximal N gene of SeV according to the method described previously [22–24] to generate recombinant SeV, SeV/SDF-1α or SeV/SDF-1β.

2.3. Northern blot

Total RNA was extracted using RNAzol-B (Tel-Test Inc., Texas) from approximately 10^6 CEF infected with the SeVSDF-1α(+) at various time points post infection (p.i.). The RNAs were ethanol precipitated, dissolved in formamide/formaldehyde solution, then electrophoresed in 1% agarose-formamide/MOPS gels, and capillary transferred onto Hibond-N filters (Amersham, UK). They were hybridized with the SDF-1 specific *Not*I fragment from pSeVSDF-1α(+) probes that had been labeled with α-P^{32}-dCTP using Multiprime DNA Labeling System (Amersham).

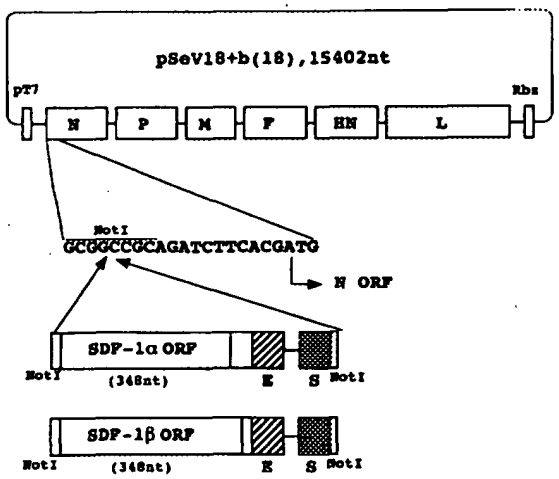

Fig. 1. Construction of the plasmids pSeV/SDF-1α(+) and pSeV/SDF-1β(+) which generate recombinant SeV/SDF-1α and SeV/SDF-1β antigenomic RNAs, respectively. The ORFs of human SDF-1α or SDF-1β followed by SeV transcriptional regulation signals E (hatched box) and S (filled box) were amplified with *Not*I-tagged primers and inserted to the *Not*I site in the parental pSeV18$^+$b(+) which generate a full length copy of the antigenomic positive sense of SeV RNA as described in Section 2.

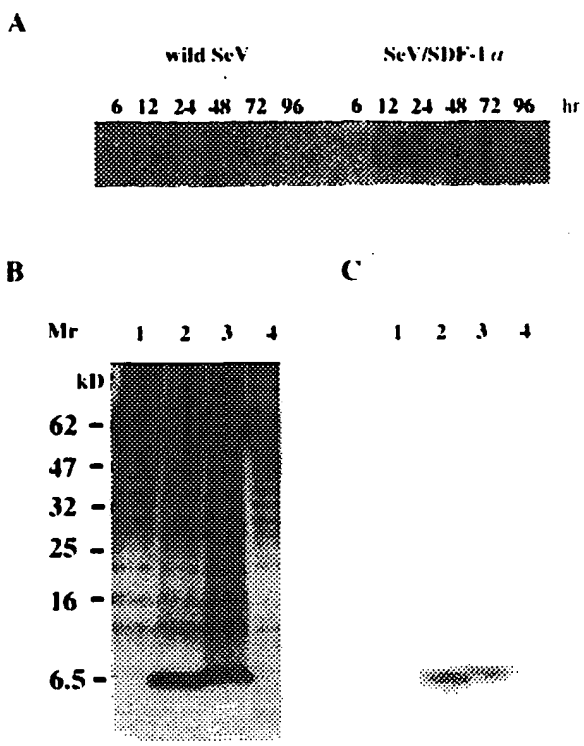

Fig. 2. Expression of human SDF-1α and SDF-1β genes from SeV/SDF-1α and SeV/SDF-1β. A: Northern blot hybridization. The RNA extracted at various hours after infection designated on the top of each lane from CEF infected with SeV/SDF-1α or wild-type SeV was analyzed by Northern blot hybridization with probe specific for SDF-1 gene. B: SDS-PAGE. Proteins in 100 μl of culture supernatant of CEF infected with wild-type SeV (lane 1), SeV/SDF-1α (lane 2), or SeV/SDF-1β (lane 3) were precipitated with ethanol together with 10 μg of BSA as carriers, and subjected to 15% SDS-PAGE. The gel was stained with Coomassie brilliant blue. Lane 4 shows 10 μg of BSA. C: Western blot analysis. Proteins resolved by SDS-PAGE were electrotransferred onto PVDF membrane and probed with anti-SDF-1 antiserum.

2.4. Western blot

Culture supernatants of infected cells were electrophoresed in 15% SDS-polyacrylamide gels [34]. The proteins in the gels were electrotransferred onto PVDF membranes (Millipore, Bedford) and probed with anti-SDF-1 antiserum, which was prepared by immunized rabbits with multiple antigen peptide containing residues 33–45 (RFFESHVARANVK) synthesized by Research Benetics Inc. (Huntsville, AL) [33].

2.5. Purification of SDF-1α and SDF-1β

Culture supernatants of CEF infected with SeV/SDF-1α or SeV/SDF-1β were harvested 72 h after infection and the SeV viruses were removed by centrifugation at 48 000×*g* for 1 h at 4°C. The supernatant was applied to an 1 ml Hi-Trap heparin column (Pharmacia, Uppsala, Sweden) equilibrated with 10 mM sodium phosphate buffer, pH 7.2, on an FPLC system (Pharmacia, Uppsala, Sweden). After washing with 5 ml of the same buffer, bound proteins were eluted with 10 ml of linear gradient of 0.4–1.0 M NaCl in the same buffer. Fractions were diluted more than 80-fold before assaying the chemotactic or anti-HIV activity. Active fractions were collected.

2.6. Chemotaxis assay

Lymphocyte chemotaxis assays were performed according to the method described by Bluel et al. [6]. Briefly, human peripheral blood lymphocytes were obtained from healthy donors by Ficoll-Histpaque method. Monocytes were removed by 1-h steps of plastic adherence.

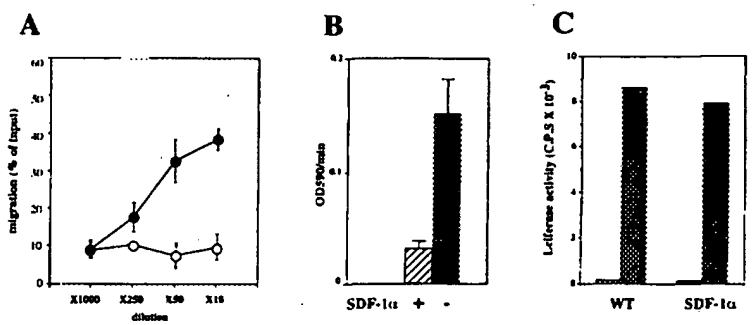

Fig. 3. Biological activities of SDF-1α. A: Chemotactic activity of SDF-1α. Culture supernatants of CEF infected with SeV/SDF-1α (○) or wild-type SeV (●) were serially diluted and examined for their chemotactic activity. Error bars indicate standard deviations of duplicated data. B: Inhibitory effect of SDF-1α on cell fusion mediated by gp160 of HIV-1 strain NL43. Filled and hatched bars indicate β-galactosidase activity within cells treated with the culture supernatants of CEF infected with wild-type SeV and SeV/SDF-1α, respectively. Error bars indicate standard deviations of duplicated data. C: Effect of SDF-1α on HIV-1 LTR-driven luciferase activity. MT4 cells were treated with culture supernatants of CEF infected with wild-type SeV (WT) of SeV/SDF-1α (SDF-1α) followed by transfection with both pHIV-1LTR/L-A-5'438 and pcDL-SRα/tat501 (filled bars), or pHIV-1LTR/L-A-5'438 alone (hatched bars). Luciferase activity within cells was assayed 40 h after transfection.

Cells (5×10^5) in 100 μl RPMI-1640 medium containing 0.25% human serum albumin (HSA) were added to the upper chamber of a 5-μm pore polycarbonate Transwell culture insert (Coaster, Cambridge, MA) and incubated with the indicated concentrations of proteins for 3 h. Transmigrated cells were counted with a FACScan (Becton Dickinson, San Jose, CA) for 20 s at 60 μl/min.

2.7. Anti-HIV-1 assay

PHA-stimulated PBMC or MT4 cells (5×10^5) were incubated with indicated concentrations of chemokines for 16 h, and then exposed to 1000 50% tissue culture infective dose of HIV-1 for 2 h at 37°C. The cells were washed twice with RPMI medium and maintained in the culture medium for each cell type. Culture supernatants of the infected cells were assayed for the levels of p24 core antigen (Abbott, Wiesbaden-Delkenheim, Germany). Data points are the means of duplicate cultures.

2.8. Cell fusion assay

A recombinant vaccinia virus-based gene activation assay using a β-galactosidase gene as a reporter was performed as described by Nussbaum et al. [35]. Briefly, L cells were transfected with plasmid pG1NT7β-gal with DOTAP and then infected with recombinant vaccinia virus expressing gp160 of HIV-1 strain NL43. MT4 cells were infected with vTF7-3 [36], and then treated with 2-fold diluted culture supernatant of CEF infected with SeV/SDF-1α or wild-type SeV. After 16-h incubation at 31°C, equal numbers (1×10^5) of L and MT4 cells were mixed and incubated at 37°C for 3 h. β-galactosidase activity within cell lysate was measured by using chlorophenol red-β-D-galactopyranoside as a substrate.

2.9. Luciferase assay

MT4 cells were incubated with or without 0.5 μg/ml of SDF-1α for 16 h, and then transfected with 5 μg of the plasmid carrying the luciferase reporter gene under the control of HIV-1 LTR, pHIV-1LTR/L-A-5'438, and 5 μg of the tat expression plasmid, pcDL-SRα/tat501, with DOTAP (Boehringer-Mannheim, Germany). Cells were maintained in the presence or absence of 0.5 μg/ml of SDF-1α for additional 40 h and then lysed for luciferase assay [22].

2.10. Ca^{2+} influx assay

Freshly prepared PBMC were stimulated with PHA for 3 days in RPMI without IL-2. Ca^{2+} influx into PHA-stimulated PBMC upon SDF-1α treatment was assayed as described previously [17].

3. Results

3.1. Generation of a recombinant SeV carrying human SDF-1α gene

The SeV genome of 15-kb long negative sense RNA is organized starting with a short 3'-leader region, followed by six

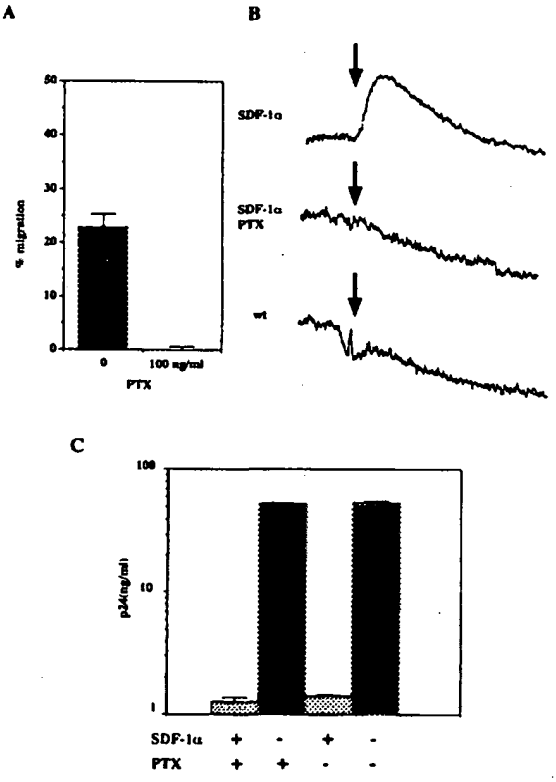

Fig. 4. A: Effect of PTX on chemotactic activity of SDF-1α. Freshly prepared PBL were incubated at 37°C for 16 h in the presence or absence of 100 ng/ml of PTX. Migration of PBL to 20-fold diluted culture supernatants of CEF infected with SeV/SDF-1α was performed as described in Section 2. Error bars indicate standard deviations of duplicated data. B: Effect of PTX on SDF-1α mediated Ca^{2+} influx into PBMC. Dialyzed culture supernatants of CEF infected with SeV/SDF-1α (SDF-1α) or wild-type SeV (wt) were added at the time point indicated by arrows. C: Effect of PTX on HIV-1 growth and SDF-1α mediated anti-HIV-1 activity. MT4 cells were incubated at 37°C for 16 h in the presence (+) or absence (−) of 100 ng/ml of PTX, and treated with culture supernatants of CEF infected with SeV/SDF-1α or wild-type SeV, and then infected with NL43 strain of HIV-1. The levels of p24 core antigen in the culture supernatants were assayed 3 days after infection. Data points are mean actual fluctuations of duplicated culture.

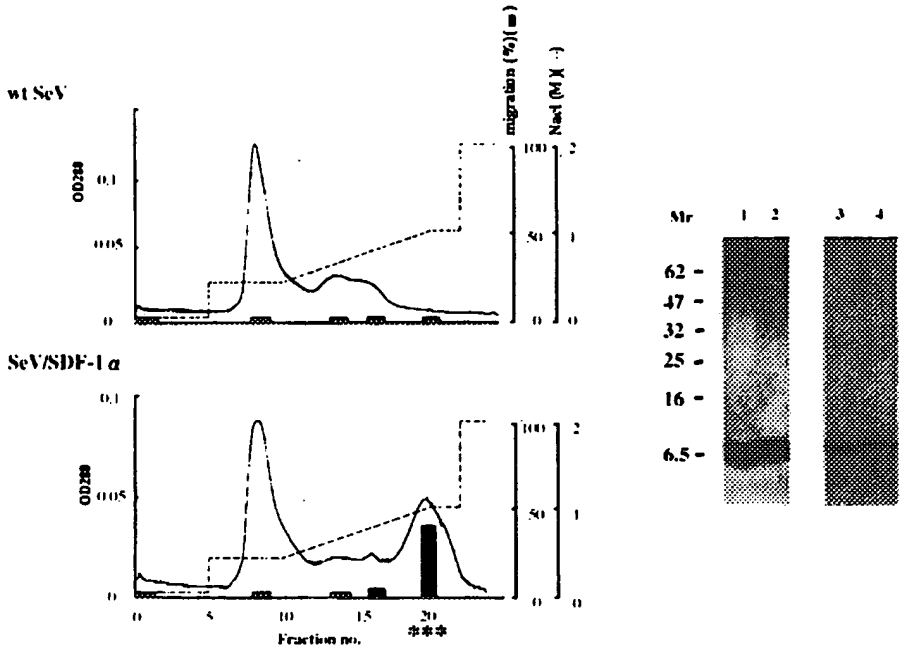

Fig. 5. Left panel: Purification of SDF-1α. SDF-1α was purified from the culture supernatant of CEF infected with SeV/SDF-1α by binding to Hi-Trap heparin column. Bars in the column chromatogram indicate chemotactic activity. Right panel: SDS-PAGE of purified SDF-1α and SDF-1β. Three active fractions shown by asterisks in the left panel were pooled and subjected to 15% SDS-PAGE. Proteins were silver-stained (lanes 1 and 2) or probed with anti-SDF-1 serum after electrotransferring to PVDF membrane (lanes 3 and 4). Positions of molecular weight markers are shown. Lanes 1 and 3, SDF-1α; lanes 2 and 4, SDF-1β.

structural genes and ending with the short 5′-trailer region [37–39]. There is only a single promoter for RNA polymerase consisting of L and P proteins [40], at the 3′ end. By recognizing the stop or end (E) (termination/polyadenylation) and restart (S) signals, the polymerase gives rise to leader RNA and each mRNA species. The plasmid pSeV18$^+$(+) carries a cDNA copy of SeV full length antigenome (positive strand RNA), in which an additional 18 synthetic nucleotides containing unique *Not*I site was inserted. This insertion was done within the first gene locus (N gene) and just upstream of its ORF, and serves as the site for further insertion of a foreign gene of interest [23]. Placing the foreign gene in this 3′-terminal first locus expects the highest expression, because of polar attenuation of gene expression toward the 5′-terminus [41]. The entire viral sequence containing the 18-nucleotide insertion was placed between the T7 promoter and the hepatitis delta virus ribozyme. The latter was used to generate a precise 3′ end.

A 348-bp DNA fragment containing entire coding frame of human SDF-1α gene (267 bp) followed by a new set of synthetic E and S signals with intervening three nucleotides was amplified with *Not*I-tagged primers and inserted into the *Not*I site in pSev18$^+$b(+), generating pSeVSDF-1α(+) (Fig. 1). In the cells infected with recovered recombinant virus, the S signal originally used for the N gene start directs the initiation of inserted SDF-1α gene transcription, and the introduced synthetic E and S signals direct the termination of inserted SDF-1α gene and the transcription initiation of the downstream N gene, respectively. pSeVSDF-1α(+) was transfected to v-TF7-3 infected LLCMK2 cells and the T7-driven full length recombinant SeV RNA genomes were encapsulated with N, P and L proteins, which were derived from the cotransfected respective plasmids. Following a 40-h incubation to allow initiation of the infectious cycle and generation of progeny, the transfected cells were injected into embryonated chicken eggs to amplify the recovered virus. After a successive passage

Table 1
Effect of SDF-1α on the growth of several HIV-1 and SIV mac strains

Strain	Phenotype	p24 (ng/ml)	
		Control	SDF-1α (0.5 μg/ml)
NL43[a]	SI/T cell line tropic	54.44	1.40
SF33[a]	SI/T cell line tropic	78.50	6.40
TK11[a]	SI/T cell line tropic	355.00	26.00
#15[b]	SI	100.23	1.36
SF162[b]	NSI/macrophage tropic	10.43	12.35
#12[b]	NSI	27.91	17.70
#37[b]	NSI	22.01	20.00
SIV mac239[a]	T cell line tropic	4.81[c]	6.22[c]

SI and NSI indicate syncytium inducing and non-syncytium inducing phenotype, respectively. Data points are means of duplicate cultures.
[a]Inhibition by SDF-1α in MT4 cells was evaluated at day 3 after infection.
[b]Inhibition by SDF-1α in PBMC cultures was evaluated at day 7 after infection.
[c]SIV mac p27 core antigen levels are shown.

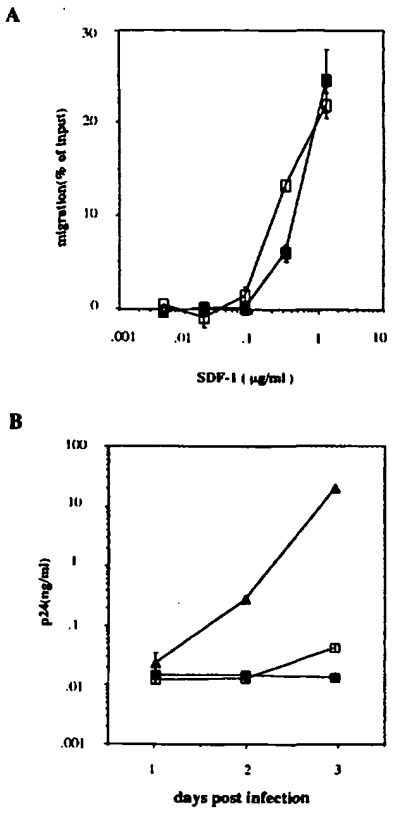

Fig. 6. A: Chemotactic activity of SDF-1α and SDF-1β. Purified SDF-1α (□) and SDF-1β (■) were serially diluted and assayed for the chemotactic activity. B: Anti-HIV-1 activity of SDF-1α and SDF-1β. MT4 cells were treated with 200 ng/ml of purified SDF-1α (□) or SDF-1β (■), and then infected with NL43 strain of HIV-1. The levels of p24 core antigen in the culture supernatants were assayed periodically. Data points are mean actual fluctuations of duplicate cultures. Filled triangles show cultures which were not treated with chemokines.

in eggs, the recombinant virus reached a titer of over 10^9 PFU/ml comparable to that of the wild-type SeV. This second passage, initiated at a dilution of 10^{-6}, resulted in complete elimination of helper vTF7-3 present in an amount of 10^4 to 10^5 PFU/ml. Nucleotide sequencing of the recombinant virus revealed that there was no accidental nucleotide substitution within the inserted SDF-1α gene. The recovered virus was named SeV/SDF-1α.

3.2. *Anti-HIV-1 and chemotactic activities of SDF-1α expressed from SeV/SDF-1*

SeV has a broad host range and reaches quite a high copy number in susceptible cells. In previous experiments, we found that expression levels were the highest in CV1, a monkey kidney cell line [23,24]. Here, we found that primary chicken embryo fibroblasts (CEF) were still more productive, and used in all subsequent studies. In CEF infected with SeV/SDF-1α, the transcripts specific to SDF-1α with an expected size of approximately 400 bases were detected (Fig. 2A), and a polypeptide with a molecular mass of 8 kDa was observed as a major protein constituent in the culture supernatant (Fig. 2B). This protein species was absent in wild-type SeV infected fluid, and reacted specifically with rabbit serum immunized with 13-mer peptides derived from human SDF-1α sequence in Western blotting (Fig. 2C). These results demonstrated that substantial amounts of SDF-1α were produced from the recombinant SeV and secreted into the culture supernatant. After a 72-h incubation, the amount of SDF-1α in the culture fluid reached over 10 μg/ml.

Because the culture fluid efficiently attracted freshly prepared human peripheral blood lymphocytes (PBL) in chemotaxis assays (Fig. 3A), the recombinant SDF-1α was functionally authentic. The recombinant SDF-1α suppressed the replication of three different T cell line tropic HIV-1 strains, NL43, SF33 and TK11, in the MT4 T cell line, and one syncytium inducing primary isolate #15 in PBMC culture (Table 1). The SDF-1α did not suppress the replication of macrophage tropic strain SF162, nor two non-syncytium inducing primary isolates, #12 and #37, in PBMC cultures (Table 1). The recombinant SDF-1α did not show any inhibitory activity for simian immunodeficiency virus (SIV) strain mac239 in MT4 cells (Table 1). These results are consistent with the expected specificity of antiviral activities of SDF-1α and thus confirmed the biological authenticity of the SeV-derived recombinant SDF-1α. We further demonstrated that the recombinant SDF-1α indeed inhibited the step of membrane fusion, but not the viral transcription (Fig. 3B and C).

3.3. *Effect of a G protein blocker on SDF-1 mediated inhibition of HIV-1 growth*

Pretreatment of PBL with 100 ng/ml of pertussis toxin (PTX) for 18 h completely abolished the chemotactic activity of SDF-1α (Fig. 4A). Ca^{2+} mobilization into PBL mediated by SDF-1α was also inhibited by this reagent (Fig. 4B), confirming that SDF-1α transduces signals mediated by Gαi-coupled receptor. However, the same concentration of PTX did not affect the inhibitory activity of SDF-1α in HIV-1 growth (Fig. 4C). The blocker alone did not affect HIV-1 replication at all. These results indicated that the G protein mediated signal transduction is neither a prerequisite for HIV-1 entry nor essential for the inhibitory action of SDF-1α.

3.4. *Purification of the recombinant SDF-1α from culture supernatants*

An affinity heparin column chromatography was performed to purify SDF-1α from the culture supernatant of SeV/SDF-1α infected CEF. The chemotactic and anti-HIV-1 activity were co-eluted with a single 8 kDa polypeptide in the chromatography (Fig. 5). We consistently obtained at least 50 μg of purified SDF-1α from 50 ml crude culture supernatant. Amino acid sequencing demonstrated the NH_2-terminal KPVSLSYRXPXR, identical to the reported sequence of SDF-1α. In this NH_2-terminal peptide, X should be read as C, because it cannot be resolved by the sequencing method employed. In the purified SDF-1α preparation, we detected a small amount of protein with the N-terminal sequence, SYRXPXRFFE, which lacked the first five amino acid residues of the SDF-1α sequence. This minor species of the NH_2-terminal sequence was also reported to be present in the SDF-1α preparation purified from a bone marrow stromal cell line [6] and has been shown to be functionally inactive in both chemotactic and HIV-1 inhibition [15].

3.5. *Comparison of SDF-1α and SDF-1β*

The human and murine SDF-1 gene gives rise to two forms, SDF-1α and SDF-1β, by alternative splicing. They differ in

the carboxy-terminal four amino acid residues, which are present in SDF-1β and absent in SDF-1α [1,4]. We also successfully constructed a recombinant SeV expressing SDF-1β (SeV/SDF-1β) (Fig. 1). As in the case of SDF-1α, the amount of SDF-1β reached over 10 μg/ml in the culture supernatant of CEF infected with SeV/SDF-1β, and readily purified by heparin column chromatography (Fig. 5). Consistent with the fact that SDF-1β has additional four amino acid residues, it migrated slightly more slowly than SDF-1α in SDS-PAGE (Figs. 2B and 5).

The chemotactic and anti-HIV-1 activities of SDF-1β were examined and compared with those of SDF-1α. SDF-1β was found to attract freshly prepared PBL in chemotaxis assay and suppressed HIV-1 strain NL43 as efficiently as did SDF-1α (Fig. 6).

4. Discussion

In this paper, we have described SeV-based expression of both SDF-1α and SDF-1β. They were of equal efficacy in chemotactic activity on freshly prepared PBL and inhibiting HIV-1 replication. Their specificity was demonstrated by selective inhibition of T cell line tropic laboratory strains and SI type primary isolates. In most previous papers on SDF-1α, it was either chemically synthesized or physiologically produced from bone marrow stromal cells [6,15,17,19,42–45]. In the latter, native molecules can be obtained in conditioned media but in an estimated amount of only 1 μg/ml at most. In the former, the primary product should be oxidized to form disulfide bridges, and then carefully purified by high performance liquid chromatography. Moreover, the production is laborious and expensive. Only a single round of synthesis of 1 mg may require numerous days and cost. In our expression system, SDF-1α as well as SDF-1β accumulated extensively, reaching as high as 10 μg/ml or more, in the culture supernatants. Thus, the product existed as a major protein constituent in the supernatant, indicating its remarkable enrichment already in the crude material. Because of this high level of production and because an amount around 200 ng/ml is sufficient for chemotaxis and antiviral assay, culture supernatants without further condensation and purification could be conveniently used as the source of SDF-1α and SDF-1β for various experiments described above. It will be thus also easy to test the functions of derivatives produced from recombinant viruses following various engineering of their genes.

SDF-1α we generated efficiently suppressed replication of T cell line tropic or primary SI strains but not macrophage tropic or NSI strains of HIV-1. It was recently reported that simian immunodeficiency virus (SIV) strains do not utilize CXCR-4 as a co-receptor, although they can grow very well in T cell lines which lack another co-receptor, CCR-5 [46–48]. Co-receptors for SIV were recently identified and named BOB/gpr1, Bonzo and gpr15 [49,50]. Consistent with these findings, recombinant SDF-1α did not suppress replication of SIV strain mac239 at all even in the MT4 T cell line.

The transcripts of murine SDF-1α and SDF-1β were observed in many tissues including the brain, heart, lung, kidney, thymus, liver, and spleen, and the amount of transcripts of SDF-1α was always higher than that of SDF-1β [1]. In the present study, we found that SDF-1α and SDF-1β were equally effective in both chemotaxis and virus inhibition. On the other hand, it is reported that negative charges in the C-terminus of CC-chemokine MIP-1α were important for its tendency to form self-aggregation [51]. Since two out of four amino acid residues specific for SDF-1β are basic lysine residues, SDF-1α and SDF-1β may differ in their tendency to form self-aggregates. Thus, SDF-1α and SDF-1β may display different rates of in vivo spread, and therefore their contribution to in vivo physiology and the effects on HIV-1 containment may differ both quantitatively and qualitatively. It will be important to learn the physical properties and antiviral actions of recombinant SDF-1α and SDF-1β in vivo.

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Characterizations of the human parainfluenza type 2 virus gene encoding the L protein and the intergenic sequences

Mitsuo Kawano[1,2], Kousuke Okamoto[1], Hisanori Bando[1], Kunio Kondo[1,2], Masato Tsurudome[1], Hiroshi Komada[1], Machiko Nishio[1] and Yasuhiko Ito[1]*

[1]Department of Microbiology, Mie University School of Medicine, 2-174 Edobashi, Tsu-Shi, Mie Prefecture 514 and [2]Fujikura Research Center, Fujikura Kasei Co. Ltd, 3-20-7 Hasune, Itabashi, Tokyo 174, Japan

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ABSTRACT

We cloned and determined the nucleotide sequences of cDNAs against genomic RNA encoding the L protein of human parainfluenza type 2 virus (PIV-2). The L gene is 6904 nucleotides long including the intergenic region at the HN-L junction and putative negative strand leader RNA, almost all of which is complementary to the p sitive strand leader RNA of PIV-2. The deduced L protein contains 2262 amino acids with a calculated molecular weight of 256,366. The L protein of PIV-2 sh ws 39.9, 28.9, 27.8 and 28.3% homologies with Newcastle disease virus (NDV), Sendai virus (SV), parainfluenza type 3 virus (PIV-3) and measles virus (MV), respectively. Although sequence data on other components of transcriptive complex, NP and P, suggested a closer relationship between PIV-2 and MV, as concerns the L protein, MV is closely related to another group as SV and PIV-3. From analysis of the alignment of the five L proteins, six blocks composed of conserved amino acids were found in the L proteins. The L protein of PIV-2 was detected in purified virions and virus-infected cells using antiserum directed against an oligopeptide corresponding to the amino terminal region. Primer extension analyses showed that the intergenic regions at the NP-P, P-M, M-F, F-HN and HN-L junctions are 4, 45, 28, 8 and 42 nucleotides long, respectively, indicating that the intergenic regions xhibit no conservation of length and sequence. Furthermore, the starting and ending sequences of paramyxoviruses were summarized.

INTRODUCTION

Human parainfluenza type 2 virus (PIV-2) is a member of the family Paramyxoviridae. PIV-2 infects respiratory tract of infant, causing croup occasionally. PIV-2 has a single-stranded, negative sense genomic RNA with approximately 15 k nucleotides and the gen mic RNA codes seven structural proteins; the nucleocapsid (NP), phospho (P), V, Matrix (M), hemagglutinin-neuraminidase (HN), fusion (F) and large (L) proteins, like other paramyxoviruses. All the genes other than the L gene of PIV-2 were sequenced and analyzed in our laboratory (1–5). The remaining uncharacterized gene, L gene, encodes a large polypeptide with a molecular weight greater than 200K which is believed to be required for transcription and replication of viral genomic RNA (6,7). There is evidence that the L proteins of vesicular stomatitis virus (VSV) may be responsible for synthesis of viral mRNA and capping, methylation, polyadenylation f newly synthesized viral mRNAs and protein kinase activity (8). In this study, we cloned the cDNAs of the genomic RNA coding for the L protein of PIV-2 and determined the nucleotide sequences. The deduced amino acid sequence of PIV-2 was compared with those of other paramyxovirus L genes reported previously (9,10,11,12). Furthermore, the L protein was detected in purified virions and virus-infected cells using antiserum directed against an oligopeptide specific for predicted L polypeptide. In addition to the PIV-2 L gene, we have also determined the intergenic sequences in the NP-P, F-HN and HN-L junctions, and summarized the intergenic, starting (R1) and ending (R2) sequences of paramyxoviruses.

MATERIALS AND METHODS

Cell and virus

Human parainfluenza type 2 virus (PIV-2), Toshiba strain, and Vero cells were used in this study. Virus propagation and purification of virus mRNA and nucleocapsid RNA were performed as previously reported (1,13).

Construction and screening of cDNA libraries

Construction methods of the cDNA libraries derived from mRNA and genomic RNA were described previously (1).

DNA sequencing and sequence analyses

Subclones were obtained from the clones containing a large insert cDNA digested with appropriate six-base-recognition restriction endonucleases. Sequencing was performed by the dideoxy chain

* To whom correspondence should be addressed

termination method (14) using the T7 DNA polymerase (US Biochemical Corp., Cleveland, OH) and [a-^{35}S]dATP (DuPont, NEN Research Products, Boston, MA). The analyses of sequence data were aided by SDC-GENETYX programs.

Primer extension

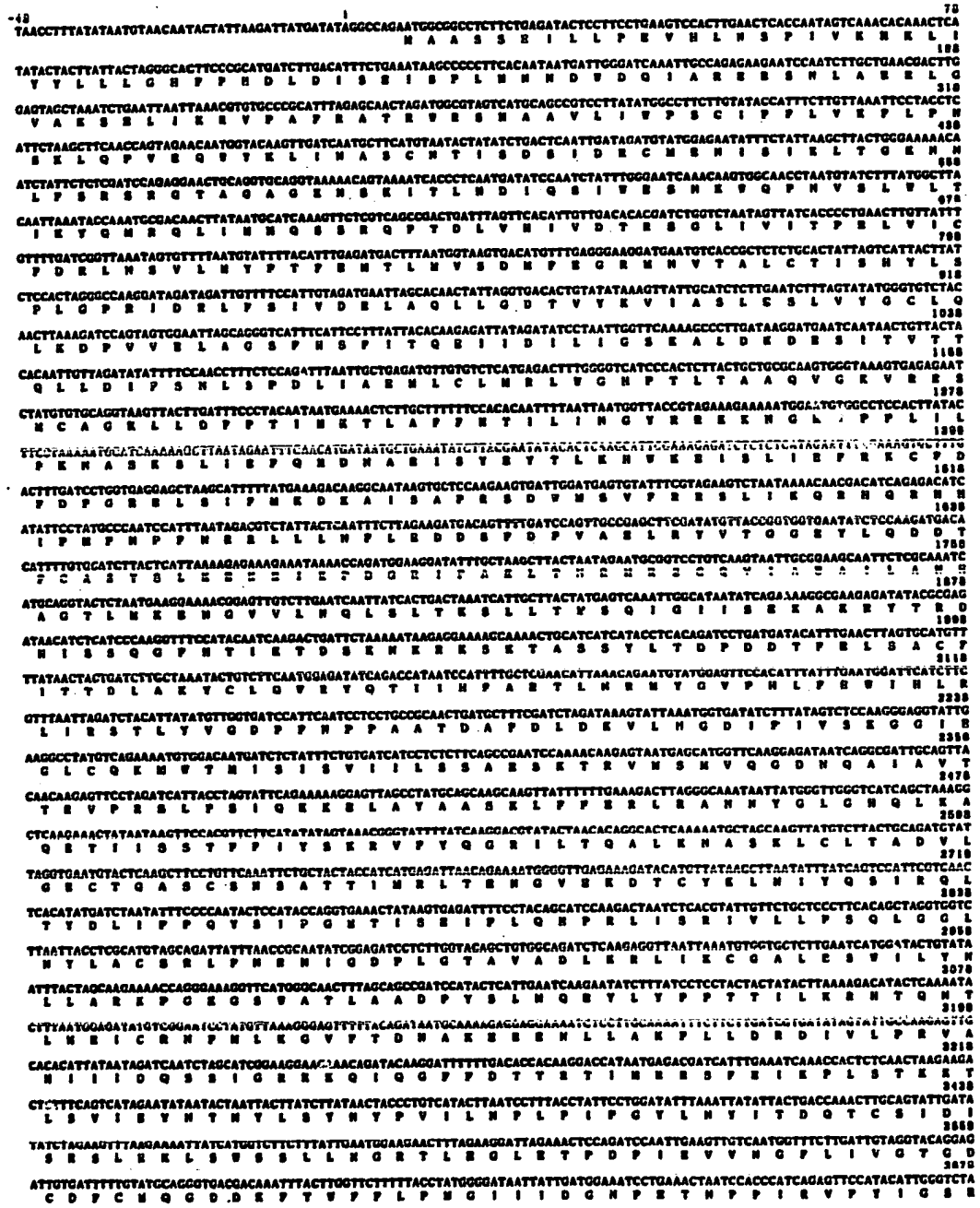
For determination of 5′ terminus of each mRNA and intergenic sequences, primer extensions from the 5′ ends of the P, HN and L genes were carried out using purified poly(A)$^+$ RNA for the templates as previously described (2).

Synthetic peptides and antibodies

One sequence, E-I-L-L-P-E-V-H-L-N-S-P-I-V-K-H-K, that corresponded to hydrophilic amino-terminal region was chosen from the predicted sequence. The sequence shows high identity with the corresponding regions of other paramyxovirus L proteins. The peptide was synthesized and conjugated with bovine serum albumin. To generate polyclonal antisera to the synthetic peptide, group of two rabbits was injected intramuscularly at multiple sites with 500ug of peptide conjugated in an emulsion of PBS and Freund's complete adjuvant. Injections were repeated every 2 weeks with the same amount of peptide in PBS and the adjuvant. Rabbits were bled before the first injection and 3 weeks after the fifth injection.

Anti-peptide assay

Rabbit sera were tested for the presence of antibodies to the inoculated peptide by the indirect enzyme-linked immunosorbent



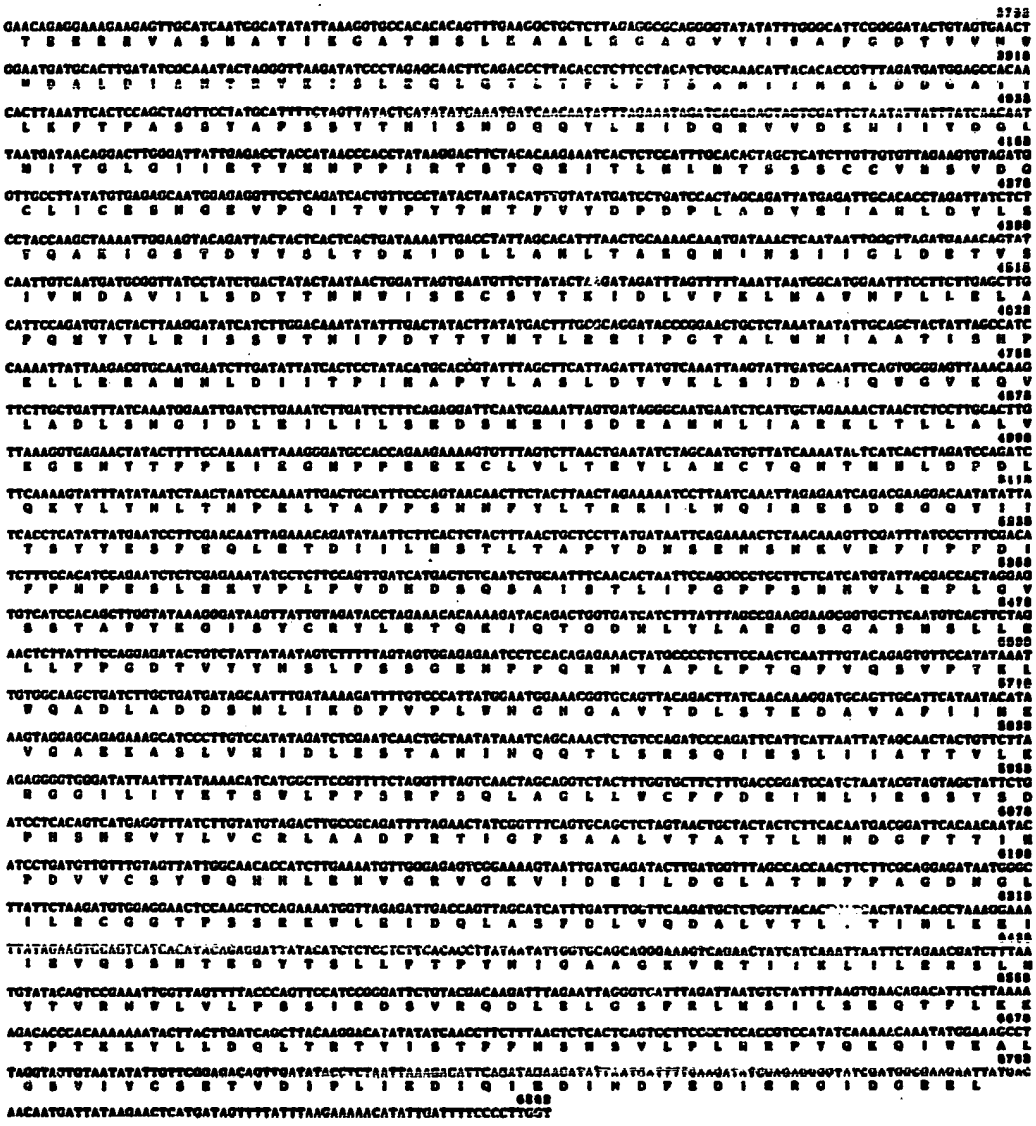


Fig 1. The nucleotide sequence of the L gene and the resulting amino acid sequence of the L protein. The nucleotide sequence is presented in the positive sense, and includes parts of the intergenic sequence at the HN-L junction, poly (A) tract and negative sense leader RNA.

assay (ELISA), as described by Tsurudome et al. (15) and Ohgimoto et al. (5). Titers of antisera were 32,000 and 2,000, respectively.

Immunofluorescence and Western blot stainings

Indirect immunofluorescence and Western blot staining methods were reported previously (16,17).

RESULTS

Nucleotide sequence of the L gene of PIV-2

The complete nucleotide sequence f the L gene f PIV-2, Toshiba strain, was determined. The L gene spans 6904 nucleotides including the intergenic region at the HN-L junction and putative negative strand leader RNA (Fig 1). Almost all the putative negative strand leader RNA is complementary to the positive strand leader RNA of PIV-2 (4). The L mRNA contains a single large open reading frame starting at the first AUG at 9 nucleotides from 5' end. The deduced protein contains 2262 amino acids with a calculated molecular weight of 256,366. The L mRNA has the following sequences: 5'AGGCCAGAAUG–L coding region–UGA–31 nucleotides–UAUUUAAG–poly (A)3'.

Protein structure of the L protein of PIV-2, and comparison of these amino acid sequences with other paramyxoviruses

In order to locate functionally homologous and unique domains in the L protein, the amino acid sequence of the PIV-2 L protein has been aligned and compared with those of Sendai virus (SV, 9), Newcastle disease virus (NDV, 10), human parainfluenza type 3 virus (PIV-3, 11) and measles virus (MV, 12) (Fig 2). Subsequently, we have calculated the percentage of identifiable amino acid sequences between the L proteins (Table 1). The L protein of PIV-2 shows 39.9, 28.9, 27.8 and 28.3% homologies with NDV, SV, PIV-3 or MV, respectively. These similarities

show high value compared with other paramyxovirus proteins, and distinct homology is detected between the L proteins of paramyxoviruses and that of vesicular stomatitis virus (VSV) belonging to rhabdovirus (data not shown). These results suggest that the L proteins of paramyxovirus and rhabdovirus share a common evolutionary history. On the basis of the alignment, a phylogenetic tree was constructed for five paramyxoviruses using the unweighted pairwise grouping (UPG) method (18)(Fig 3). As shown in Fig 3, paramyxoviruses are divided into two large groups; one consists of PIV-2 and NDV, and another group consists of SV and PIV-3. Transcriptive complex of paramyxovirus is composed of NP, P and L proteins (19). Data on the sequences of NP and P proteins suggested that MV, PIV-2 and NDV were grouped with (4,20). Interestingly, as concerns the L protein, MV belongs to the same group as PIV-1 and PIV-3.

This alignment shows that high identities exist over almost the entire length of the proteins. However, in the C-terminal third of the L proteins of the paramyxoviruses there is no significant amino acid identity. 295 of amino acids are conserved among all the five viruses and high conservation of Gly residues among the invariant amino acids may be related to the functional and structural importance of these residues in these L proteins. According to dot matrix plot (data not shown), six blocks (A to F) composed of conserved amino acids were found in all the L proteins (Boxed in Fig 2). The blocks of the highest amino acid conservation (C to F) are located in the central region of the protein. A careful analysis of these aligned blocks indicates that blocks A and B consist mainly of hydrophobic residues but carboxy terminus of block B is flanked by hydrophilic residues. Charged amino acids are predominant in blocks C and D. In block C, a central highly conserved stretch is very rich in basic amino acids. Long conserved stretches including invariant hexapeptide VQGDNQ are detected throughout block D. There are 6 invariant Pro residues and the other longest continuous stretch of invariant amino acids (RNIGDP) in Block E. Block F contains five Cys and three His residues. Almost all the conserved Cys and His residues exist in this block.

Detection of the L protein in the purified PIV-2 virions and in the PIV-2 infected cells

Purified PIV-2 virions were subjected to gel electrophoresis, blotted onto a nitrocellulose membrane and immunostained with antiserum directed against an oligopeptide corresponding to an amino-terminal region of the PIV-2 L protein, which shows high identity with other paramyxoviruses. As shown in Fig 4, one band (about 220K) reacted with the antiserum. The extract did not react with preimmunized rabbit serum. The antiserum also reacted with the L protein of NDV (data not shown).

In next experiment, the synthesis of the L protein and its distribution in infected cells were examined by indirect

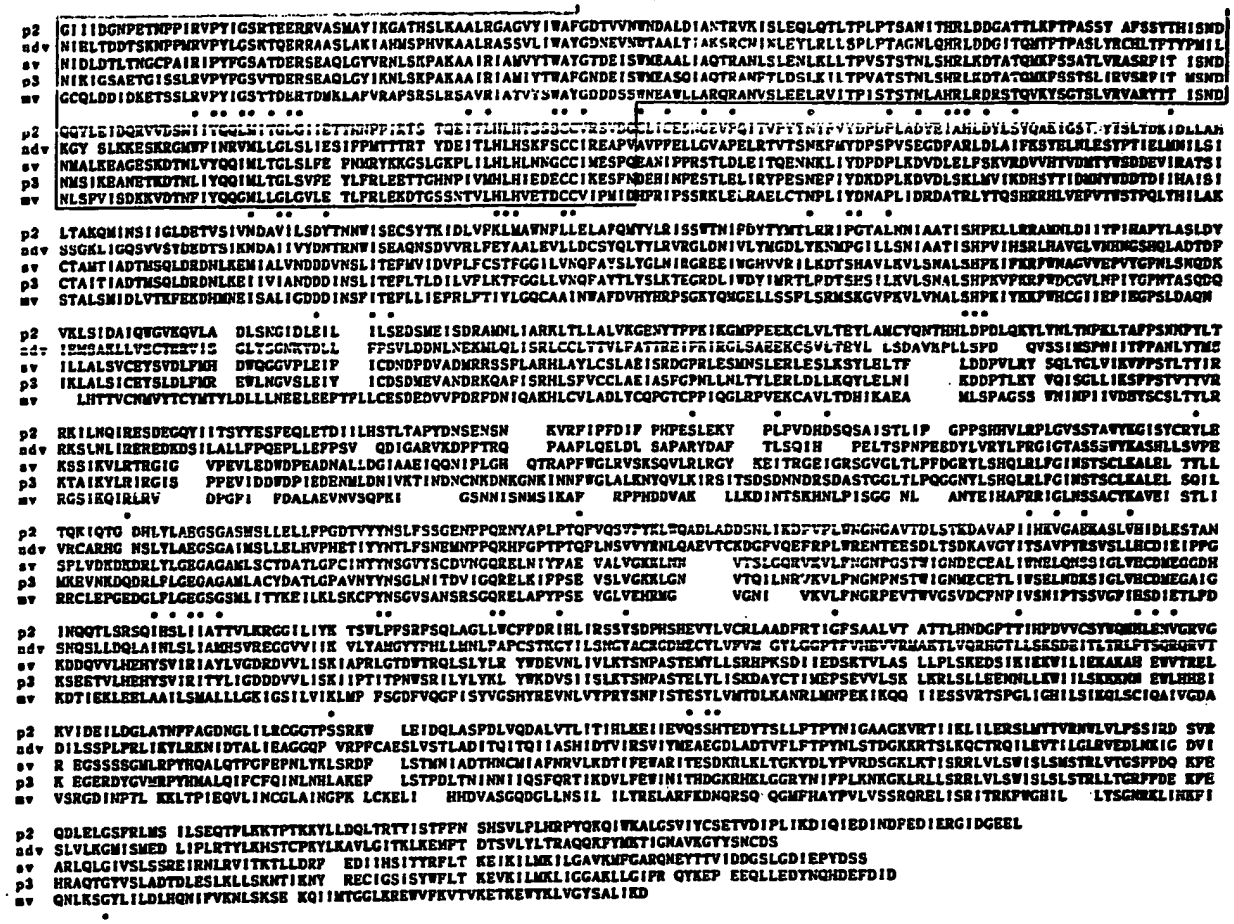


Fig 2. Sequence alignment of five paramyxovirus L proteins, PIV-2, NDV (10), PIV-3 (11), SV (9) and MV (12). Asterisks under the amino acid sequences denote the residues common to all five viruses and boxes show the conserved domains.

Table 1. Percentage identity of amino acid sequences between the L proteins of paramyxoviruses

Virus	NDV	SV	PIV-3	MV
PIV-2	39.9	28.9	27.8	28.3
NDV		24.7	25.0	24.1
SV			58.9	42.4
PIV-3				37.4

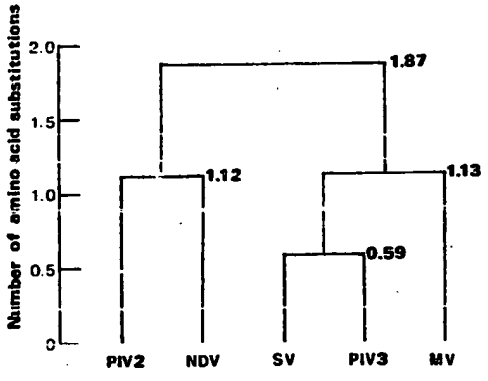


Fig 3. The phylogenetic tree for the L proteins of paramyxoviruses. On the basis of the alignment (Fig 2), we estimated the number of amino acid substitutions for all pairs (Table 1) and constructed phylogenetic trees for five paramyxoviruses using the unweighted pairwise grouping (UPG) method.

immunofluorescence staining using the antiserum described above and monoclonal antibody directed against the PIV-2 NP. At 6, 12, 16, 20 and 24 hours after incubation of PIV-2-infected Vero cells, the cells were fixed with cold acetone at 4C for 5 min. Faint florescence was observed in the cytoplasm at 16 hours post-infection (p.i.), and thereafter fluorescence was more intensive and showed large granules (Fig 5B). However, fluorescence observed in anti-NP antibody-stained cells at 12 hours p.i. was stronger than that in anti-L antibody-stained cells at 24 hours p.i. (data not shown).

Intergenic, gene-starting and -ending sequences of PIV-2

The intergenic sequences at the P-M and M-F junctions were reported previously (2,3). Thus, remaining intergenic sequences at the NP-P, F-HN and HN-L junctions were determined using primer extension method. The results are presented in Fig 6. Furthermore, starting, ending and intergenic sequences f paramyxoviruses are summarized (Fig 6). The intergenic sequences at the HN-L junction of PIV-2 is AUUGGAAAUA-UAUUACAUUGUUAUGAUAAUUCUAAUACUAUA

(negative sense). This sequence is extremely long compared with the sequences of other paramyxoviruses. However, the intergenic sequences at the P-M and M-F junctions are also long, 45 and 28 nucleotides, respectively, which are similar to the intergenic sequences of the corresponding junctions of SV 5 (20)(Fig 6). The intergenic sequence at the F-HN junction is GGAUUUUA, which is very similar to the intergenic sequence, GAUUUUA, at the F-SH (small hydrophobic gene) junction of MuV (21). The gene-starting and -ending sequences of the L mRNA of PIV-2 are UCCGGUCU and AUAAAUUC, respectively. The starting sequence of the L gene is similar to those of the P and F genes, but is different from those of the NP and HN genes. On the contrary, the ending sequences is highly conserved in all PIV-2 genes and shows strong identities with the gene-ending consensuses of other paramyxoviruses.

DISCUSSION

Human parainfluenza viruses are divided into two groups: PIV-1 and -3 belong to one group, while PIV-2, -4A and -4B belong to the other group(13,22,23). These groups are immunologically and genetically distinct from each other. Almost all the genes other than L gene of paramyxoviruses have been sequenced, while informations about L genes and L proteins are relatively few. The L genes of the former group were sequenced and analyzed, but that of the latter group has not been sequenced yet. In this study, the complete nucleotide sequence of the PIV-2 L gene was determined and analyzed.

On a basis of alignment of five L proteins of paramyxoviruses, six blocks of conserved amino acids were identified. The L proteins of paramyxovirus is probably multifunctional in the processes of viral transcription and replication, that is, initiation, elongation, termination, polyadenylation, capping, methylation, kinasion and so on. A hypothesis that each block identified in the L protein may correspond to each function is worth considering. Subsequently, we searched for particular oligopeptide motifs implicated in RNA polymerase function of the L protein. Kamer and Argos (24) have initially identified a consensus sequence (DD, usually preceded by a G) flanked by hydrophobic residues to be present in a number of known and putative RNA-dependent RNA polymerase of plant, animal and bacterial viruses. This motif has been found in segmented negative-strand RNA viruses (PB1 and PB2 polymerases of influenza virus), positive-strand RNA viruses (poliovirus, sindbis virus), and retrovirus (human immunodeficiency virus, Rous sarcoma virus). The number and location of this motif is, however, widely variable in all paramyxovirus L proteins including PIV-2. Only one of these DD motif is located at identical position with that of NDV.

Interestingly, the common stretch of amino acids (QGDNQ), another variant motif of the GDD motif, found in all the L proteins reported previously is also detected in the PIV-2 L protein. Since the stretch is flanked by hydrophobic residues in all the L proteins, QGDNQ and its neighboring sequences may constitute an active site of paramyxovirus L proteins. A primary requirement of all L proteins is their capacity to interact with template RNA. Therefore, template recognition sites are expected to be positively charged-domains and common regions among paramyxoviruses. Such a domain may be represented by the highly conserved central region of block C. This site is constituted by the clusters of invariant basic amino acids (Lys, Arg). Basic amino acids interspersed with nonbasic ones are believed to constitute the binding domains in a variety of RNA binding proteins (25,26). The purine nucleotide-binding sites consist of a Lys residue neighboured by numerous Gly residues (27). In a number of protein kinases, consensus of ATP binding site has been identified as a GxGxxG--15 to 20 amino acids--K motif (28). GxxGxGK(S/T) motif was found in purine-binding proteins (29). An analogous glycine-rich motif (K--GxGxG) is shared by three

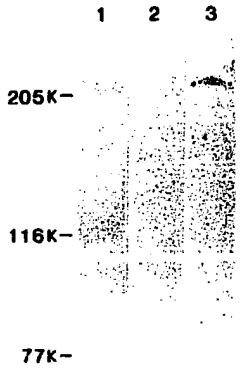


Fig 4. Detection of the L protein in purified virions by Western blot assay with anti-L specific oligopeptide antiserum (#1,titer: 32,000). The purified PIV-2 virions were subjected to 9% polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose membrane and tested for antibody binding by the Western blot method. Lane 1, molecular weight markers. Lane 2, preimmune serum. Lane 3, postimmune serum.

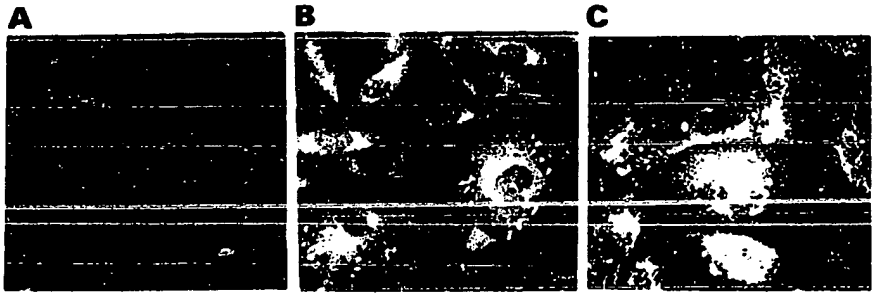


Fig 5. Indirect immunofluorescent antibody staining of mock- and PIV-2-infected Vero cells. Vero cells were grown and incubated in the presence of monkey serum. Mock- and PIV-2-infected cells were fixed with cold acetone. A: mock-infected cells. B and C: PIV-2-infected cells at 20 hours p.i. A and B: anti-L specific oligopeptide antiserum. C: MAb directed against the NP.

L proteins of SV, PIV-3 and MV near C-terminus in the L proteins, but a conserved downstream Lys residue is missing. On the contrary, in the L protein of PIV-2 and NDV belonging to other group, alanine is substituted for the first Gly. Poch et al. (8) have proposed that this site could be associated with polyadenylation r with protein kinase activities. Other glycine-rich motif (GGIEG--K---R) exists in Block D, following Arg in place of conserved downstream Lys.

Mapping of the 5′ termini of the P, HN and L mRNAs was carried out and these intergenic sequences at the NP-P, F-HN and HN-L junctions were determined using primer extension. Furthermore, the intergenic, starting and ending sequences of paramyxoviruses were summarized. The intergenic region between the NP and P genes was found to be 4 nucleotides long, while those at the P-M, M-F, F-HN and HN-L junctions were numerous, 45, 28, 8 and 42 nucleotides, respectively. These findings were similar to those of SV 5 and NDV; the intergenic regions at the P-M and M-F junctions of SV 5 were 15 and 22 nucleotides (20) and the intergenic sequences at the F-HN and HN-L junctions of NDV were found to be 31 and 44 nucleotides (10). Therefore, there is no consensus in either length or sequence of intergenic regions of PIV-2, SV 5, MuV (21) and NDV, all f which belong to same group. The intergenic sequence at the PIV-2 F-HN junction is GGAUUUUA, which is very similar t the intergenic sequence, GAUUUUA, at the F-SH (small hydrophobic gene) junction of MuV (21). In previous study (2), it was clearly shown that the 3′-end of the SH gene of MuV corresponded to the 3′-end of the HN gene of PIV-2 and the first nucleotide of the HN gene of MuV corresponded to the nucleotide 83 of the PIV-2 HN gene, suggesting that deletion or insertion might have occurred between the F and HN genes during a long evolutionary time. The similarity between the intergenic sequences at the F-HN junction of PIV-2 and the F-SH junction of MuV confirms the previous speculation. On the other hand, the intergenic sequences within SV and PIV-3 genomes (30), which belong to the other group of paramyxovirus, were trinucleotide and GAA is the most popular trinucleotide. The intergenic sequences of MV genome(31) is also almost the same trinucleotide, and the striking identity between SV, PIV-3 and MV suggests that these viruses share a common ancestor. The intergenic regions are thought to be the site of transcriptional attenuation and contain the signal for termination of transcription. What distinct difference of conservation degree in the sequence and length observed between groups of paramyxovirus means remains unclear.

In PIV-2, the 5′ terminal sequences (R1) of the mRNAs are not conserved, while R1 of SV, PIV-3 and MV are well conserved. Since R1 is thought to be the transcriptional initiation signal, there is less of a constraint against variation in the gene-starting signal of PIV-2 in comparison to other paramyxoviruses, implying a gene-specific regulation or the low dependence f transcription initiation of PIV-2 polymerase on the primary sequence. On the contrary, the ending sequence was conserved in all PIV-2 genes and showed identities with the gene-ending consensuses of other paramyxoviruses, indicating the existence of a common attenuation mechanism.

virus	gene-end	poly(U) tract	intergenic sequence	gene-start
piv2				UCUAAGCCACGG <NP>
muv				UUCGGUCCUUCA <NP>
piv3				UCCUAAUUUCUG <NP>
sv				UCCCAGUUUCAU <NP>
mv				UCCUAAGUUCUA <NP>
piv2	<NP> UUAAAUUC	UUUUUU	AGUA	UCCGGCCUGCCC <P>
sv5	<NP> GAAAUUUC	UUUUUUUU	••	ACCGGCCUGCCC <P>*
muv	<NP> AGAAAUUC	UUUUUU	AA	UCCGGGCCUUUC <P>
piv3	<NP> AUUUAUUC	UUUUU	GAA	UCCUAAUUUCUU <P>
sv	<NP> CAUCAUUC	UUUUU	GAA	UCCCACUUUCAA <P>
mv	<NP> UAACAAUA	UUUUUU	GAA	UCCUUGCUCC <P>
piv2	<P> UUAAAUUA	UUUUUUU	CUUUUCUUCAAUUCAUUUUAAAUUUCCUGUGUUAUCUCUUUUAGA	UCCAGGCUUUCG <M>
sv5	<P> CCAAAAUC	UUUUUU	GCUAAUUGCUAUUUA	UUCGGGCUUCUG <M>
muv	<P> AUAAUUUA	UUUUUU	A	UUCGUGCUUGUG <M>
piv3	<P> GUUUAUUC	UUUUU	GAA	UCCUAAUUUCUU <M>
sv	<P> UCUAAUUC	UUUUU	GAA	UCCCACUUUCUU <M>
mv	<P> AGGUAAUA	UUUUUU (U)	GAA	UCCUCGUUUC <M>
piv2	<M> UUAGAUUG	UUUUUUU	GAUUUGCAAGGUGUUAUUUAGUUGCAAG	UCCGGUUUUAUA <P>
sv5	<M> UAAGUUUC	UUUU	GUUUAGUAUAAAUCUGAUAGCA	UUCGUGCUUGGG <P>
muv	<M> UUAAUAUC	UUUUUU	A	UUCGGAUCUUCC <P>
piv3	<M> UCUAUUAG	UUUUU	GAA	UCCUGUUUUCUU <P>
sv	<M> AUUUAUUC	UUUUU	GAA	UCCCUAUUUCAG <P>
mv	<M> UUGAUUUG	UUUU	GAA	UCCCGCUUCC <P>
piv2	<P> UUAAAUUC	UUUUUU	GGAUUUUA	UUCGUGCUUCGG <HN>
sv5	<P> AAAAAUUC	UUUUUUU	GCUA	UCCUGGCUUGGA <SH>
muv	<P> UCUAAAUC	UUUUUUU	GAUUUUA	UUCUUACUUAGA <SH>
piv3	<P> UUUUAAUA	UUUUUU	GAA	UCCUCAUUUCAA <HN>
sv	<P> UAUUAUUC	UUUUU	GAA	UCCCACUUUCAC <HN>
mv	<P> UCAAUUAA	UUUU	GAA	UCCCACGUUC <HA>
sv5	<SH> AAAAUUUC	UUUUUU	AU	CCGGGCUUGUGU <HN>
muv	<SH> UGUAUUUC	UUUUUU	CG	UUCGGUCUUGUC <HN>
piv2	<HN> AUAAAUUC	UUUUUU	AUUGGAAAUAUAUUACAUUGUUAUGAUAAUUCUAAUACUAUA	UCCGGUCU <L>
sv5	<HN> CAAAAUUC	••		
muv	<HN> AAUAAUUC	UUUUUU	G	UCCGGUCUUACC <L>
piv3	<HN> UCUUUAUA	UUUUUU	GAA	UCCUCGUUUCU <L>
sv	<HN> CAUAAUUC	UUUUU	GGG	UCCCACUUACCC <L>
mv	<HA> CUUAAUUC	UUUUU	GCA	UCCCACGUUC <L>
piv2	<L> AUAAAUUC	UUUUU		
piv3	<L> UUUCAUUC	UUUUU		
sv	<L> UAUCAUUC	UUUUU		
mv	<L> AUAAUUUC	UUUU		

Fig 6. Summary of the gene-ending, intergenic and gene-starting sequences of paramyxoviruses. *From our unpublished sequence data, it was suggested that the intergenic sequence of the NP-P junction and the gene starting sequence of P gene of SV 5 were A and UCCGGCCUGCCC, respectively.

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AUTHOR(S): Amara, Rama Rao (1); Ma, Marjgery; Smith, James (1); Grimm, Bennett; McNicholl, Janet; Villinger, Francois; Altman, John; Robinson, Harriet (1)
CORPORATE SOURCE: (1) Yerkes Primate Research Center, Atlanta, GA USA
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213 Pertussis Toxin B-oligomer as a Novel and Potent Anti-HIV Agent.

M. Alfano[1], G. Vallanti[1], P. Biswas[2], C. Bovolenta[1], E. Vicenzi[1], R. Rappuoli[3], A. Lazzarin[2], N. Sidenius[1], F. Blasi[1], T. Pushkarsky[4], M. Bukrinsky[4], and G. Poli[1].
[1]DIBIT. and [2]Lab. of Clinical Immunology, San Raffaele Scientific Institute, 20132 Milan, ITALY, [3]IRIS Research Center, Chiron SpA, 53100 Siena, ITALY, [4]The Picower Institute for Medical Research, 11030 Manhasset, New York, USA.

The B-oligomeric subunit of pertussis toxin (B-ol) specifically inhibits entry and replication of R5 and replication of X4 HIV-1 strains in primary T lymphocytes (Alfano M. et al, J. Exp. Med., 190:597-605, 1999). Here, we show that B-ol also blocks post-entry events in the T cell line Jurkat, and that this mechanism is Tat dependent. In monocyte-derived macrophages (MDM), inhibition by B-ol mostly occued at post-entry steps as proved by using HIV pseudotyped with ADA, MLV or VSV-G envelope. To further investigate the mechanisms of HIV-1 post-entry inhibition by B-ol we used a chronically infected promonocytic cell line, in which virus expression is triggered by PMA or multiple cytokines. A reduction of multiply spliced and unspliced HIV RNAs was observed in U1 cells stimulated with TNF-a in the presence of B-ol although, surprisingly enough, the activation of cellular trascription factor NF-kB was unaffected. B-ol also suppressed cell-associated viral proteins and virion production in U1 stimulated with IL-6, a cytokine predominantly acting on HIV expression at post-trascriptional level.
B-ol effects in U1 cells are likely to be mediated by the integrin MAC-1, one of the three known B-oligomer receptors, and ongoing experiments are aimed at addressing this point.
Since the mutant pertussis toxin, PT-9K/129G, already safely administered to humans for prevention of *Bordetella Pertussis* induced disease, shows an anti-HIV profile superimposable to that of B-ol, at least *in vitro*, B-ol may be a novel and potent inhibitor of HIV-1 replication to be tested in infected individuals.

214 Generation of High Frequency CD4 and CD8 Positive T Cell Responses by Priming DNA Expressing Gag-Pol-Env (VLP) or Gag-Pol (Gag particles) Followed by Recombinant MVA Boosters

Rama Rao Amara[1], Marjgery Ma[2], James Smith[1], Bennett Grimm[2], Janet McNicholl[2], Francois Villinger[3], John Altman[4] and Harriet Robinson[1].
[1]Yerkes Primate Research Center, [2]Centers for Disease Control, [3]Emory School of Medicine, [4]Vaccine Research Center at Yerkes Atlanta, Georgia.

CD8 positive T cells have been shown to be very effective in containment of HIV replication. We have taken "DNA prime, recombinant MVA boost" approach for efficient generation of CD8 as well as CD4 positive T cells specific for HIV proteins. Rhesus macaques were vaccinated against SHIV 89.6 using the DNA encoding HIV 89.6 envelope as well as SIV gag, pol proteins or SIV gag, pol proteins that produce Virus Like Particles (VLP). Two different routes of immunization (i.e. intradermal and intramascular) two different doses of immmogen (i.e. 2500 and 250 micrograms, respectively) as well as the effect of co-immunization with DNA expressing GM-CSF were studied during the priming of the response. The presence of env in the VLPs is believed to enhance receptor mediated uptake of these particles which may result in efficient priming of CD8 positive T cell response. We compared the advantage of presence of env protein in the VLP by vaccinating animals using a DNA expressing all the genes that are present in the VLP except the env which can still form the particles (GAG particles). Animals were subsequently boosted with recombinant MVA expressing all the genes that were present in the priming DNA. The frequency of antigen specific CD8 positive T cells were measured by Tetramer staining, Intracellular Cytokine Staining (ICC) and ELISPOT assays before and after the recombinant MVA booster. High dose intradermal DNA injection followed by recombinant MVA boost resulted in the expansion of p11C-Tetramer positive CD8 positive T cells to as high as 19%. A dose response was observed and was in direct correlation with the dose. Intradermal injections were better than intramascular injections. During priming VLPs seemed to have primed a better response compared to GAG particles. However after rMVA boost not much differences were observed between these two immunogens. ICC analysis revealed that these CD8 as well as CD4 positive T cells produce interferon gamma. ELISPOT analysis with peptide pools specific for gag showed that T cell response was generated to various epitopes across the gag protein. Experiments are in progress to look at the ability of these T cells to protect from a SHIV 89.6P challenge.

215 Inhibition of SIV gag Particles Assembly at the Plasma Membrane by gag Products Translated from Internal Methionine Codons.

S. Arnoud, J. Zhang, M. Nermut,[1] R. Wattiez,[2] B. Charloteaux,[3] R. Brasseur,[3] A. Burny, F.Bex.
Department of Molecular Biology, University of Brussels, Belgium; 1. National Institute for Biological Standards and Control, UK; 2. Department of Biological Chemistry, University of Mons-Hainaut, Belgium; 3. Faculty of Agronomy of Gembloux, Belgium.

Simian immunodeficiency viruses (SIV) predominantly assemble at the plasma membrane. This process is driven by the Gag polyprotein precursor. To study the process involved in the targeting of SIV virus particles, the Gag polyprotein from two SIV strains, SIVmacJ5, a slow inducer of AIDS, and SIVsmmPBj14 which induces an acute wasting syndrome, were expressed in mammalian cells by a Semliki Forest virus vector. Electron and confocal microscopy indicated that the Gag precursors of these two SIV strains were targeted to different cellular membranes. The PBj14 Gag precursor formed budding virus like particles at the plasma membrane and in intracellular vesicles whereas the J5 Gag precursor was essentially found as incomplete particles in the endoplasmic reticulum. Lack of J5 plasma membrane associated particles correlated with the expression of amino-terminal truncated Gag forms initiated at internal methionine codons. Co-expression of the full size Gag together with truncated Gag forms led to retargeting of the full-length precursor to intracellular membranes. A mutant with substituted internal methionine codons assembled and budded at the plasma membrane emphasizing a dominant role of the internal methionine codons in the targeting process. Competition at the assembly site or for a transport system or both could explain our observations.

216 The Enzymatic Activity of Cholera-Like Enterotoxins Is Responsible for the Activation of Dendritic Cells *in vitro* and for Blocking the Induction of Oral Tolerance.

Ken C. Bagley, Sayed F. Abdel-Wahab, Timothy R. Fouts and George K. Lewis
Institute of Human Virology, University of Maryland at Baltimore, 725 W. Lombard Street, Baltimore Maryland, 21201

Cholera toxin (CT) and *E. coli* heat-labile enterotoxin (LT) are powerful mucosal adjuvants that block the induction of oral tolerance. There is emerging evidence that monocyte-derived dendritic cells (MDDC) are activated by these toxins but little is known about how this occurs. We investigated the role of the enzymatic activity of these toxins in their ability to activate MDDC *in vitro* and to block the induction of oral tolerance. MDDC were exposed to wild type CT and LT, the ADP-ribosylation defective mutants (CTK63 and LTK63) and their recombinant B subunits (rCTB and rLTB). CT and LT increased the surface expression of activation markers as detected by flow cytometry. By contrast, the enzymatically inactive mutants and the recombinant B subunits failed to increase these markers. In addition, MDDC treated with CT induced higher proliferation of antigen specific naive T cells than untreated MDDC *in vitro*. To address the role of the enzymatic activity of these toxins in their ability to block the induction of oral tolerance, BALB/C mice were orally primed with OVA with or without CT, LT, CTK63, LTK63, rCTB or rLTB, followed by feeding of large doses of OVA. The ability of the above constructs to block the induction of oral tolerance was assessed by anti-OVA antibody titers in the sera. CT and LT, but not CTK63, LTK63, CTB or LTB, blocked the induction of oral tolerance. Taken together, these observations indicate that an enzymatically active A subunit of CT and LT is necessary for both their toxic effects and their immunostimulatory effects.

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5. センダイウイルスベクターを用いたSDF－1aの効率的発現に関する研究

分担研究者： 塩田 達雄（東京大学医科学研究所文部教官助手）
共同研究者： 長澤 岳志（大阪母子保健総合医療センター研究所）
加藤 篤、守屋 智草、永井 美之（東京大学医科学研究所）

研究要旨
昨年、長い間未知であったHIV-1感染に必須なコレセプターがMIP-1αなどのケモカインのレセプターであること、ケモカインがgp120と競合的にHIV-1の細胞への吸着を阻害すること、が明らかにされた。本研究は、ケモカインの中でも大量に得ることが困難で解析の進んでいないSDF-1を大量発現し、抗HIV活性を詳しく検討することを目的とする。このため我々はヒトSDF-1α遺伝子をセンダイウイルスに組み込み、HIV-1NL43株の増殖に及ぼす影響を検討した。その結果、この組み換えセンダイウイルスはMT4株化T細胞でHIV-1NL43株の増殖を強く阻害し、機能的なSDF-1が産生されていることが明らかになった。

A.研究目的

昨年、長い間未知であったHIV-1感染に必須なコレセプターがケモカインのレセプターであること、ケモカインがgp120と競合的にHIV-1の細胞への吸着を阻害すること、が明らかにされた。マクロファージ指向性のHIV-1はβ-ケモカインのMIP-1α、MIP-1β、RANTESなどに、株化T細胞指向性のHIV-1はα-ケモカインのSDF-1によってその増殖が阻害されることが報告されており、ケモカインあるいはその誘導体を用いた新たな抗HIV療法の開発が期待されている。しかしβ-ケモカインがすでに合成ペプチドや大腸菌を用いた発現系で大量に作製され、解析が進んでいたのに対し、α-ケモカインのSDF-1は今日まで大量発現が困難で解析が遅れていた。本研究は、センダイウイルスベクターを用いてSDF-1を大量に発現させ、抗HIV活性を詳しく検討することを目的とする。

B.研究方法

SDF-1遺伝子：マウスより得られたSDF-1α遺伝子をヒト型に改変して用いた。
HIV-1：株化T細胞指向性の実験室株としてNL43株を使用した。
組み換えセンダイウイルスの作製：センダイウイルス(SeV)ゲノム上に存在する6つの遺伝子のうち、ゲノム3'端に位置し最も発現量の多いNP遺伝子のさらに3'側に、SDF-1α遺伝子を挿入した。DNAクローンからの感染性SeVの回収は、作製したDNAクローンと、SeVのRNA合成を行うNP、P、およびL蛋白質遺伝子を発現するプラスミドとを、あらかじめT7RNAポリメラーゼを発現するワクシニアウイルスに感染したLLCMK2細胞にトランスフェクションすることにより行った。感染性SeVが得られたか否かの検定は、トランスフェクション後48時間目に細胞を発育鶏卵に接種し、72時間培養してから、漿尿液中の赤血球凝集反応価を測定することにより行った。

C.研究結果

SDF-1は現在までに抗体が得られていない。そのため、SDF-1が発現されたかどうかを検定するためにはSDF-1の生物活性を

指標にする以外に方法がない。そのため、我々はまず、株化T細胞MT4にSDF-1を組み込んだSeVをmoi20で感染させてから24時間後、HIV-1NL43株をMoi0.05で感染させ、培養上清中のHIV-1p24抗原の量を経時的に測定した。その結果、HIV-1感染後72時間では、野性型SeVの場合、約20ng/mlのp24が検出されたのに対し、SDF-1を組み込んだSeVでは0.5ng/ml以下であり、HIV-1の増殖が著しく阻害されたことが明らかとなった。

ケモカインは一般に高濃度では凝集して不溶性になってしまうことが知られている。組み換えSeV感染細胞の培養上清中にはSDF-1が可溶性の画分に存在しているか否かを確認するため、SeV感染MT4細胞とHIV-1感染MT4細胞とを0.5μmのフィルターで隔てた状態で、HIV-1増殖を検討した。その結果、細胞同士の接触がおこらなくてもHIV-1の増殖は強く阻害され、SDF-1が可溶性の画分に存在していることが強く示唆された。さらにSeV感染MT4細胞の培養上清から超遠心でSeV粒子を除き、新しいMT4細胞を20時間処理してからHIV-1を感染させても、HIV-1増殖が強く阻害された。このことから、組み換えSeVの感染細胞の培養上清中にはSDF-1が可溶性の状態で存在していることが明らかになった。

また、SDF-1は株化T細胞指向性のHIV-1の増殖を抑制する活性とともに、リンパ球に対して強い走化作用を示すことが知られている。SDF-1を組み込んだSeVの感染細胞の培養上清には調整したばかりのヒト末梢血リンパ球に対して走化作用が認められ、活性のあるSDF-1が産生されていることが確認された。

D.考察

今日まで大量発現が困難で解析の遅れていたSDF-1をセンダイウイルスベクターを用いて発現させることを試み、抗HIV活性とリンパ球走化作用のあるSDF-1を作製することに成功した。抗体の入手不可能な現在、産生量の正確な見積りは困難であるが、生物活性から換算して、組み換えSeV感染細胞の培養上清中に約100ng/ml、感染発育鶏卵の尿液中には約1000ng/ml程度産生されているものと考えられる。発育鶏卵中ではストローマ細胞の培養上清の約10倍の生物活性が得られており、この系でSDF-1の大量精製が可能であると考えている。現在、ヘパリンのアフィニティーカラムでの精製を試みている。また、抗HIV-1活性のみを残し、そのほかの生物活性を欠失させた変異体（アンタゴニスト）の作製も試みている。

E.結論

今日まで大量発現が困難で解析の遅れていたSDF-1をセンダイウイルスベクターを用いて発現させることを試み、抗HIV活性とリンパ球走化作用のあるSDF-1を作製することに成功した。

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Sendai virus C proteins are categorically nonessential gene products but silencing their expression severely impairs viral replication and pathogenesis

Kurotani, Atsushi; Kiyotani, Katsuhiro; Kato, Atsushi; Shioda, Tatsuo; Sakai, Yuko; Mizumoto, Kiyohisa; Yoshida, Tetsuya; **Nagai, Yoshiyuki**

Department of Viral Infection, Institute of Medical Science, University of Tokyo, Tokyo, 108, Japan

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mRNA of Sendai virus (**SeV**), a prototypic member of ly Paramyxoviridae in the Mononegavirales superfamily ng a large no. of nonsegmented neg. strand **RNA** encodes a nested set of accessory proteins, C', C, Y1 and red to collectively as C proteins, initiating, resp., at d AUGs/114, 183, 201 in the +1 frame relative to the ORF of ?) protein, the smaller subunit of **RNA** . Among them, C is the major species expressed in ells at a molar ratio which is several-fold higher than hree. However, their function has remained an enigma. even been established whether or not the C proteins are or viral replication. Many other viruses in ales encode C-like proteins, but their roles also remain d. By taking advantage of a recently developed reverse tem to recover infectious **SeV** from cDNA, we nts in which C protein frames were variously silenced. es which did not express C and C', but did express Y1 severely attenuated in replication in tissue culture ous species and tissues, as well as in embryonated More notably, they were almost totally inca tively in-and hence nonpathoge ne expression and ge (-) vi

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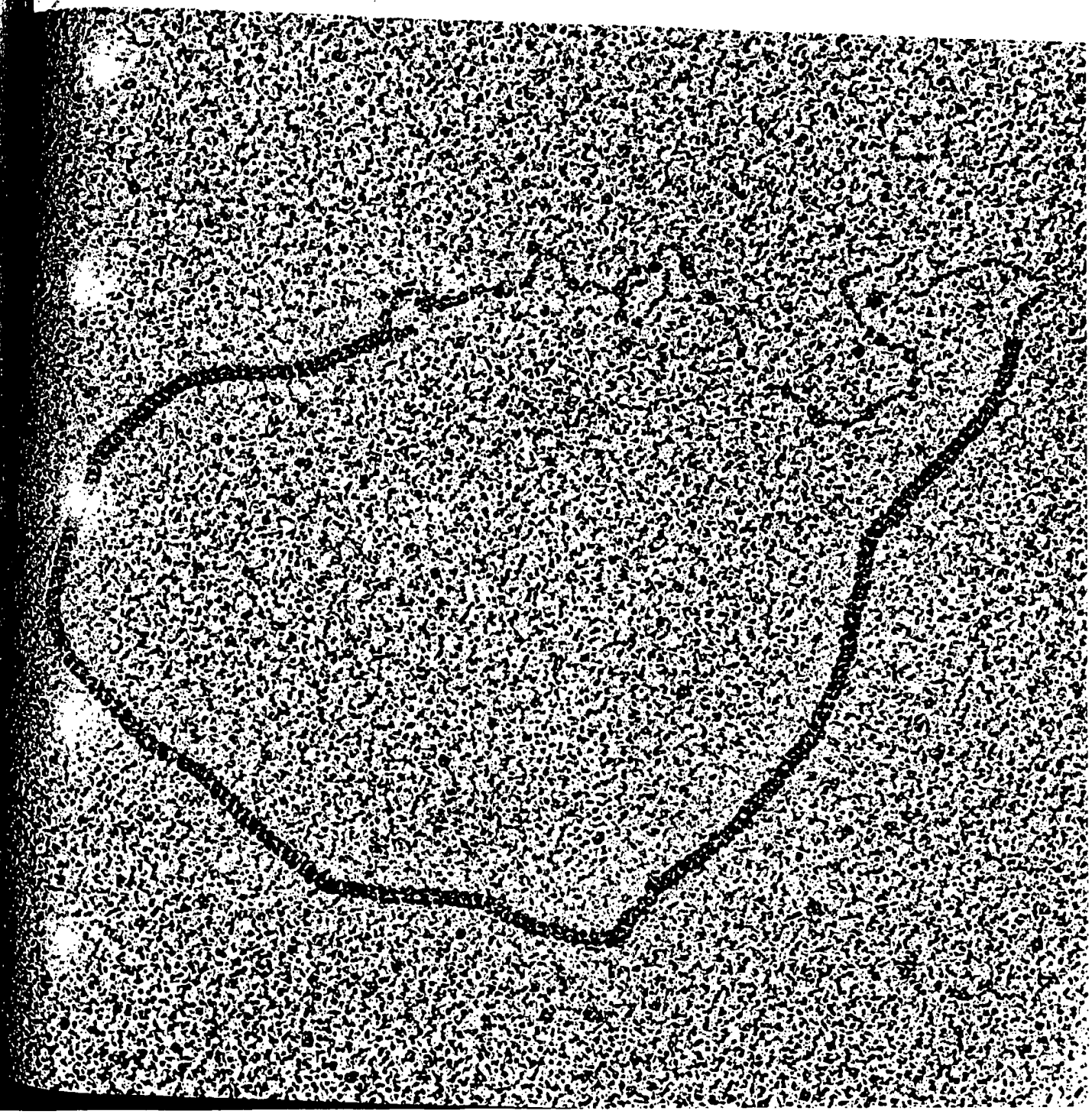

Sendai virus C proteins are categorically nonessential gene products but silencing their expression severely impairs viral replication and pathogenesis

Atsushi Kurotani[1,2,†], Katsuhiro Kiyotani[3,†], Atsushi Kato[1,†], Tatsuo Shioda[1], Yuko Sakai[1], Kiyohisa Mizumoto[2], Tetsuya Yoshida[3] and Yoshiyuki Nagai[1,*]

[1] *Department of Viral Infection, Institute of Medical Science, University of Tokyo, Shirokanedai 4-6-1, Minato-ku, Tokyo 108, Japan*
[2] *School of Pharmaceutical Sciences, Kitasato University, Shirokane 5-9-1, Minato-ku, Tokyo 108, Japan*
[3] *Department of Bacteriology, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734, Japan*

Abstract

***Background*: The P/C mRNA of Sendai virus (SeV), a prototypic member of the family *Paramyxoviridae* in the *Mononegavirales* superfamily comprising a large number of nonsegmented negative strand RNA viruses, encodes a nested set of accessory proteins, C′, C, Y1 and Y2, referred to collectively as C proteins, initiating, respectively, at ACG/81 and AUGs/114, 183, 201 in the +1 frame relative to the ORF of phospho (P) protein, the smaller subunit of RNA polymerase. Among them, C is the major species expressed in infected cells at a molar ratio which is several-fold higher than the other three. However, their function has remained an enigma. It has not even been established whether or not the C proteins are essential for viral replication. Many other viruses in *Mononegavirales* encode C-like proteins, but their roles also remain to be defined.**

***Results*: By taking advantage of a recently developed reverse genetics system to recover infectious SeV from cDNA, we created mutants in which C protein frames were variously silenced. C/C′(−) viruses which did not express C and C′, but did express Y1 and Y2, were severely attenuated in replication in tissue culture cells of various species and tissues, as well as in embryonated chicken eggs. More notably, they were almost totally incapable of growing productively in—and hence nonpathogenic for mice—the natural host. Both gene expression and genome replication appeared to be impaired in C/C′(−) viruses. Additionally silencing the Y1 and Y2 expression was also possible, and a critically impaired but viable clone, the 4C(−) virus, was isolated which expressed none of the four C proteins.**

***Conclusion*: SeV C proteins are categorically nonessential gene products, but greatly contribute to full replication capability *in vitro* and are indispensable for *in vivo* multiplication and pathogenesis. This study represents the first comprehensive functional assessment of the accessary C protein for *Mononegavirales*.**

Introduction

The nonsegmented negative-strand RNA genome of 15384 nucleotides typifies Sendai virus (SeV) as a member of the superfamily *Mononegavirales*. It belongs to the genus *Paramyxovirus* of the subfamily *Paramyxovirinae* in the family *Paramyxoviridae*. The genome encodes in a 3′ to 5′ order the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase (HN) and large protein (L). There is only a single promoter at the 3′ end for the polymerase consisting of the L and P proteins (Hamaguchi *et al.* 1983). By recognizing the stop (termination/polyadenylation) and restart signals present at each gene boundary, the polymerase gives rise to each mRNA (reviewed in Lamb & Kolakofsky 1996). The gene expression is usually monocistronic, generating a single mRNA which directs a single

Communicated by: Akira Ishihama
* *Correspondence*: E-mail: ynagai@ims.u-tokyo.ac.jp
† The first three authors contributed equally to this work.

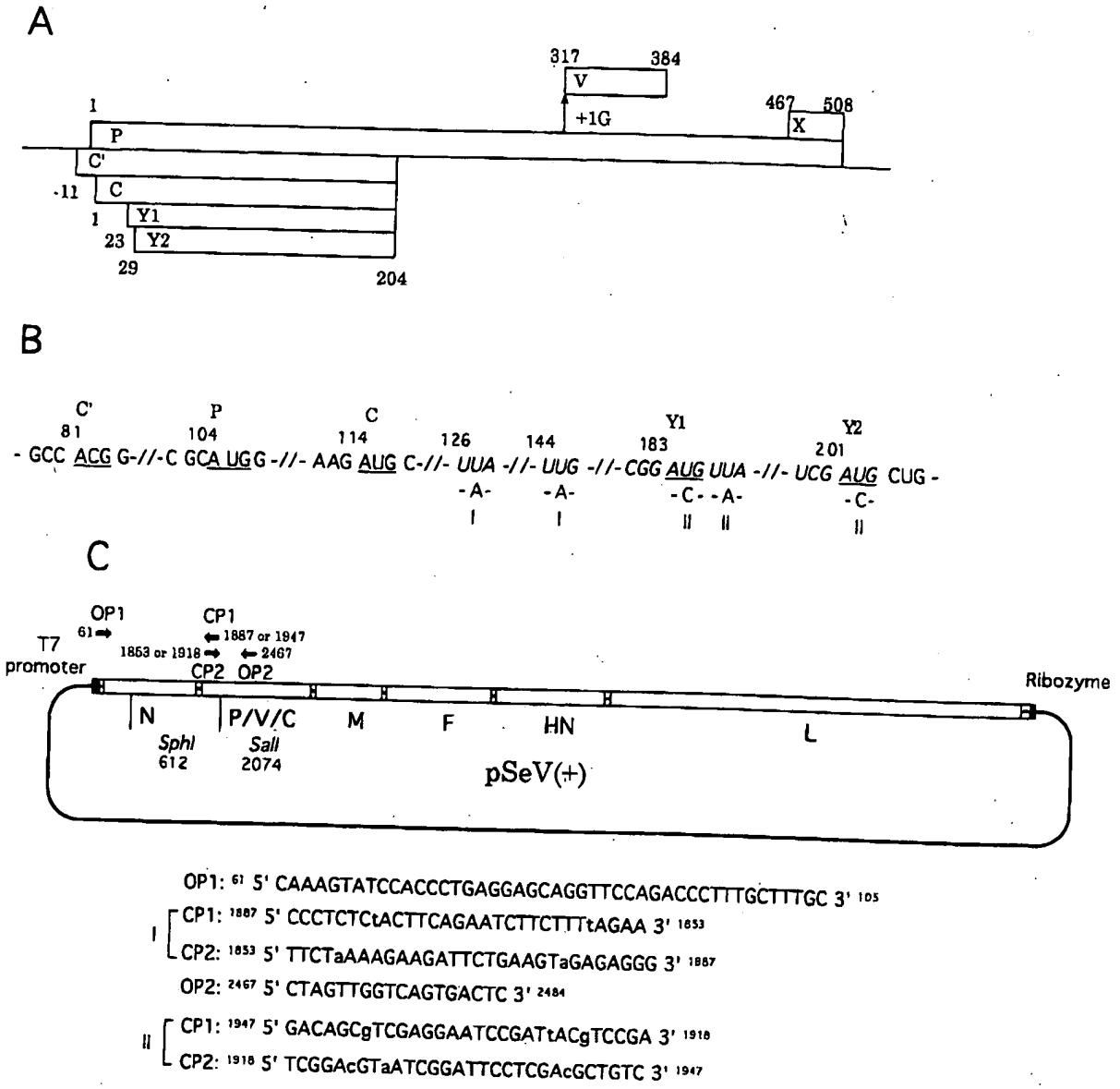

Figure 1 Mutagenesis of C ORF in the SeV P gene, (A) Coding strategies of the P gene employing overlapping frames and insertio of pseudo-templated 1 G. For details see the text. (B) Sequence around the multiple initiation codons (underlined) in P mRNA and mutagenesis to silence the expression of C-related proteins. Mutagenesis I and II were attempted. For details, see Experimental procedures. (C) Mutagenesis I and II by two-step PCR-based overlap primer extension with the respective pairs of complementary primers (CP1 and CP2) and the outer primers OP1 and OP2. The mutations in the complementary primers are shown by lower case letters. The amplified products were digested with *SphI* and *SalI*, and inserted to the corresponding region in the parental pSeV(+) which generates a full length copy of Sev antigenome RNA. For details, see Experimental procedures.

primary translation product. However, the P gene of *Paramyxovirinae* is a notable exception, because it gives rise to multiple protein species by means of overlapping frames and by a process known as RNA editing or pseudotemplated insertion of nucleotides into the transcript (reviewed in Lamb & Paterson 1991 and Lamb & Kolakofsky 1996).

In SeV RNA editing, the pseudotemplated addition of one G residue at a specific genome locus produce an mRNA that encodes the protein termed V, while the unedited mRNA that is the exact copy of the P gene encodes the P protein. Thus, the P and V proteins are amino-coterminal, while the −1 frame is used to generate the carboxy-terminal half of the V protein.

This carboxyl-terminal half is characterized by many cysteine residues, which are highly conserved among paramyxoviruses. By disrupting the editing locus in a cDNA plasmid generating a full-length copy of SeV antigenome RNA, we succeeded in recovering a virus which was defective in G insertion and V protein production. Although categorized as a nonessential gen product completely dispensable for viral replication in cells in culture, the V protein was essential for the maintenance of a high viral load in mice, the natural host, and manifest pathogenicity characterized by severe pneumonia (Kato *et al.* 1997a). Furthermore, this 'luxury' function has been primarily mapped to the unique cysteine-rich carboxyl-terminal half (Kato *et al.* 1997b).

An ORF that overlaps the amino-terminal portion of ıe SeV P ORF in the +1 frame produces a nested set of proteins which are carboxy-coterminal, called C′, C, Y1 and Y2. They are referred to collectively as the C proteins (Curran & Kolakofsky 1988a, 1989) (Fig. 1A). Translation of C′ is initiated on a non-AUG codon, ACG/81, whereas the other three start on AUGs at positions 104, 183 and 201, respectively (Curran & Kolakofsky 1988a; Gupta & Patwardhan 1988) (Fig. 1B). Ribosomes and initiation factors are pr posed to bind at or near the 5′ end of a eukaryotic mRNA in a process facilitated by the 5′ cap structure. Then they migrate in a linear manner on the mRNA, scanning until reaching the first AUG codon (Kozak 1981). The consensus nucleotide sequence containing the AUG codon for the initiation of translation is 5′-CCA/GCCAUGG-3′ (Kozak 1986), where the A or G at the −3 position and the G at position +4 (both underlined) have been found to be critical for efficient in iation (reviewed in Kozak 1989). In SeV P/C mRNA, the first ACG for C′ functions presumably because it lies in an excellent context (GCCACGG), but works poorly because of the ACG in place of AUG (Fig. 1A). The next AUG for P is in a poor context (CGCAUGG), and the third AUG for C may be in a better but still not perfect context (AAGAUGC). Thus, it is possible that some ribosome pre-initiation complexes bypass the upstream initiation codons (leaky sc nning, reviewed in Kozak 1989) and reach the AUGs for Y proteins (reviewed in Lamb & Paterson 1991). However, one additional protein called X has to be synthesized from the P frame, whose initiation codon is positioned more than 1500 nucleotides downstream of the 5′ end (Fig. 1A) (Curran & Kolakofsky 1987). It has therefore also been postulated that the synthesis of a total of six proteins is beyond the capability of leaky scanning (Lamb & Paterson 1991). Indeed, scanning-independent but cap-dependent, internal initiation has been proposed for the synthesis of Y1, Y2 and X proteins (Curran & Kolakofsky 1988b, 1989).

The C-related proteins are expressed from the P gene of all members of the genus *Paramyxovirus* and *Morbilivirus* but not *Rubulavirus*. No C proteins exist in the subfamily *Pneumovirinae*, either. Their number, expressed from these viral P genes, varies due to the use of a variable number of in-phase start codons; there are four from SeV, two or three from human parainfluenza virus type 1 (hPIV-1), but only one from hPIV-3 and measles virus P genes (reviewed in Lamb & Kolakofsky 1996). In common, the C proteins are relatively small (180–204 residues) and highly basic. Even in distantly related rhabdovirus vesicular stomotitis virus, a C-like ORF overlapping the P ORF is present, and two C proteins are expressed from this frame (Spiropoulou & Nichol 1993).

The SeV C protein was originally found in virus-infected cells, and as it was apparently absent in virions, it was thought to be a nonstructural protein (Lamb & Choppin 1977). However, subsequent observations indicated that it is detectable in small quantities in both virions and nucleocapsids isolated from cells and virions (Portner *et al.* 1986; Lamb & Paterson 1991). The SeV C protein was previously found to inhibit viral mRNA synthesis (Curran *et al.* 1991, 1992). More recently, C protein was also found to inhibit the amplification of the SeV mini-genome in cells (Cadd *et al.* 1996; Tapparel *et al.* 1997). This inhibition appeared to be promoter-specific, because the inhibitory action was exerted on an internally deleted defective interfering (DI) genome but not on a copy-back DI genome. Thus, a presumable role of C protein would be to down-regulate both genome replication and mRNA synthesis to levels which are optimal for viral replication and/or to increase replication selectivity toward optimal replication.

Here, we used SeV reverse genetics to create mutant viruses in which C protein frames are silenced, in order to address the question of how C proteins contribute to the viral life cycle *in vitro* and viral pathogenesis *in vivo*. Silencing of C′ and C frames, but not Y1 and Y2 expression suggested that C proteins up-regulate viral RNA synthesis, greatly contribute to tissue-culture replication, and are indispensable for multiplication and pathogenesis in mice. Despite such strong dependency of both *in vitro* and *in vivo* replication on C proteins, it was possible to further silence Y1 and Y2 frames and to create a critically attenuated, but viable clone in which all four C proteins were knocked out, indicating that

Table 1 Infectious virus recovery from mutated pSeV(+)

Mutation	Clone	Passage in eggs: Inoculum cell number	Virus titre (HAU) at passage: 1	2	3	4	5	Name of recovered virus
I	a	10^6	<2	16	16 (2.3×10^8)	16	NT	C/C′(−)a
		10^5	<2	<2	NT	NT	NT	
	b	10^6	<2	16	16 (1.7×10^8)	16	NT	C/C′(−)b
		10^5	<2	<2	NT	NT	NT	
	c	10^6	<2	16	16 (2.1×10^8)	16	NT	C/C′(−)c
		10^5	<2	<2	NT	NT	NT	
I+II	a	10^7	<2	<2	<2 (7.2×10^4)	4 (9.8×10^5)	8 (7.0×10^6)	4C(−)
	b	10^7	<2	<2	<2 (<10)	NT	NT	
None	a	10^5	1024	1024	1024 (1.7×10^{10})	1024	NT	wild-type

Each mutation corresponds to that shown in Fig. 1B. Infectivites are shown in parenthesis by PFU/mL for 4C(−) and CIU/mL for others. One CIU is nearly equivalent to the one PFU. NT, not tested.

SeV C proteins are categorically nonessential gene products.

Results

Recovery of C/C′(−) viruses from cDNA

The plasmid pSeV(+) generates a full-length antigenome RNA of SeV (Kato *et al.* 1996), and was used throughout this study. Site directed mutagenesis by two-step PCR-based overlap extension (Ho *et al.* 1989) was performed to terminate C ORF without changing the amino acid sequence of the P protein by using pSeV(+) as the template. For this, we introduced not one but two point mutations to convert the leucine codons at positions 5 and 11 to the termination codons UAA and UAG, respectively (mutagenesis I in Fig. 1B), in view of a possible reversion to the wild-type due to the error-prone nature of RNA virus polymerases. This manipulation would result in almost total deletion (200 residues, 93%) of the 215-residue-long C′ protein. After digestion with *SphI* and *SalI*, the mutagenized PCR product replaced the corresponding region of SeV(+) (Fig. 1C, also see Experimental procedures).

LLCMK2 cells infected with vaccinia virus vTF7-3 to express T7 polymerase were transfected with the mutagenized or wild-type pSeV(+), together with three plasmids to supply N, P and L proteins. After a 40 h incubation at 37 °C, followed by three cycles of freezing and thawing, the cells were injected into the allantoic cavity of chick embryos to amplify the recovered viruses. In all three independent attempts of mutagenesis and transfection, viruses were successfully rescued and named C/C′(−)a, b and c, respectively (Table 1). However, it was after two successive passages in eggs that their rescue could be confirmed by low haemagglutination (HA) titres, in contrast to the w d-type virus rescue capable of assessing after a single passage with a full HA titre. Furthermore, their rescue rates, which were estimated from the number of transfected cells required for virus production in eggs, were about 10-fold higher than those of the wild-type (Table 1). The viruses were once again passaged in eggs at a sufficiently high dilution to eliminate the helper vTF7-3 present in amounts of 10^4–10^5 PFU per mL, and stocked. Remarkably, all these C/C′(−) vir ses reached titres of only about 1:100 in eggs, compared with that of the wild-type (Table 1), in contrast to our previous V(−) mutants, whose titres in eggs were comparable to that of the wild-type (Kato *et al.* 1997a,b). The entire P gene of each recovered mutant virus was reverse-transcribed, PCR-amplified, and sequenced. The introduced mutations were retained in all three mutant viruses. However, in contrast to our previous manipulations for mutagenesis and for ign gene insertion, in which few unexpected accidental mutations occurred (Kato *et al.* 1996, 1997a,b; Yu *et al.* 1997; Hasan *et al.* 1997; Sakaguchi *et al.* 1997), this time several were found in the C/C′(−) viruses. These were a Glu(17) to Gly change in the P frame in C/C′(−)a due

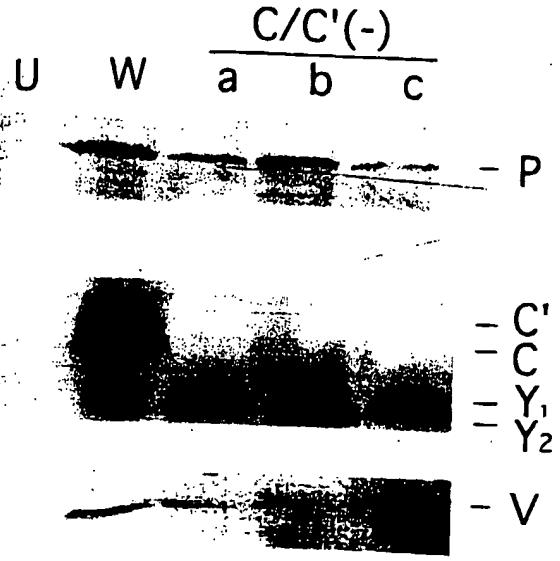

Figure 2 Western blot analysis of total protein lysates from CV1 cells infected with the wild-type SeV and three C/C′(−) viruses, a, b and c, with anti-C, anti-P and anti-V rabbit sera. Cells were infected at a multiplicity of 5 CIU/cell and harvested at 38 h p.i.

to an A(1892) to G change, Ala(58)Gly in the C frame in C/C′(−)b due to C(2025)G, and Glu(17)Gly, Ser(313)Leu, Lys(457)Glu and Arg(522)Gly in the P frame of C/C′(−)c due to A(1892)G, C(2780)T, A(3214)G and A(3409)G, respectively. There was no change that could have affected the editing signal for 1G insertion and the subsequent V frame. Because of these unexpected changes, which differed between the rescued viruses, we used all three mutants in parallel in the following studies, rather than selecting one of them. As shown above and below, however, all three viruses were almost identical in replication capability in cells in culture, in eggs and in mice. Thus, the above listed mutations did not give rise to any particular phenotype and appeared to be neutral under conditions where the expression of C/C′ was silenced.

To ascertain that the C/C′ reading frame had really been silenced, Western blot analyses were carried out using total protein lysates from CV1 cells infected with the C/C′(−) viruses. Equivalent amounts of the total proteins were separated by SDS-PAGE, blotted, and probed with anti-C, anti-P and anti-V rabbit sera as previously described (Kato *et al.* 1997a). Both the C and C′ proteins were indeed found to be knocked out in all three mutant viruses, while Y1 and Y2 were expressed (Fig. 3). Interestingly, the levels of Y1 and Y2 were much higher than those of the wild-type SeV. This latter result provides a remarkable case in which translation at an internal AUG codon in mammalian cells can be initiated or facilitated by the introduction of a termination codon within the upstream ORF (Hughes *et al.* 1984; Liu *et al.* 1984; Kozak 1984; see Discussion). It should be further noted that facilitation was more significant for the Y2 than for the Y1, in agreement with the concept that the efficiency of reinitiation steadily improves as the distance from the upstream ORF increases (Kozak 1987). Immunoblotting further confirmed that the mutagenesis did not affect the P and V frames (Fig. 2). Their expression levels were considerably lower than those of the wild-type because of the attenuated gene expression due to C/C′ knockout (see below). Taking this into account, the enhanced expression of Y1 and Y2 from the mutant viruses were even more notable. The levels of P protein and others appeared to be slightly higher in C/C′(−)b than in C/C′(−)a or C/C′(−)c (Fig. 2). This could simply be a result of fluctuation of loading quantities on SDS-PAGE, because cellular protein bands such as actin were similarly more intense (not shown).

Impaired replication of the C/C′(−) mutants in tissue-culture cells

Under single cycle growth conditions, the replication of C/C′(−) mutants was analysed in cells of three different origins, CV1 (a monkey kidney line), L929 (a mouse fibroblast line) and primary chick embryo fibroblasts (CEF). The results obtained were strikingly similar between the three C/C′(−) mutants, and were representatively shown with C/C′(−)a virus (Fig. 3A). The growth of this mutant, compared with that of the wild-type, was significantly retarded and the peak titre was reduced by 10–100-fold. The C/C′(−) viruses also featured a considerably lower cytopathic effect (CPE) on CV1 cells (Fig. 3A). The same tendency was found for L929 and CEF, but no clear-cut scoring of CPE was possible, even in infections with the wild-type virus. A multiple replication of SeV requires the presence of trypsin in the culture medium, because of a lack of endogenous protease activating the precursor Fo glycoprotein (Homma & Ohuchi 1973; Nagai 1993). Because trypsin severely damages L929 cells, multiple-step growth could only be studied with CV1 and CEF. Under multiple cycle conditions, again, the C/C′(−)a was found to exhibit much slower replication kinetics, with a markedly reduced final titre (Fig. 3B).

These results indicated that C and/or C′ proteins

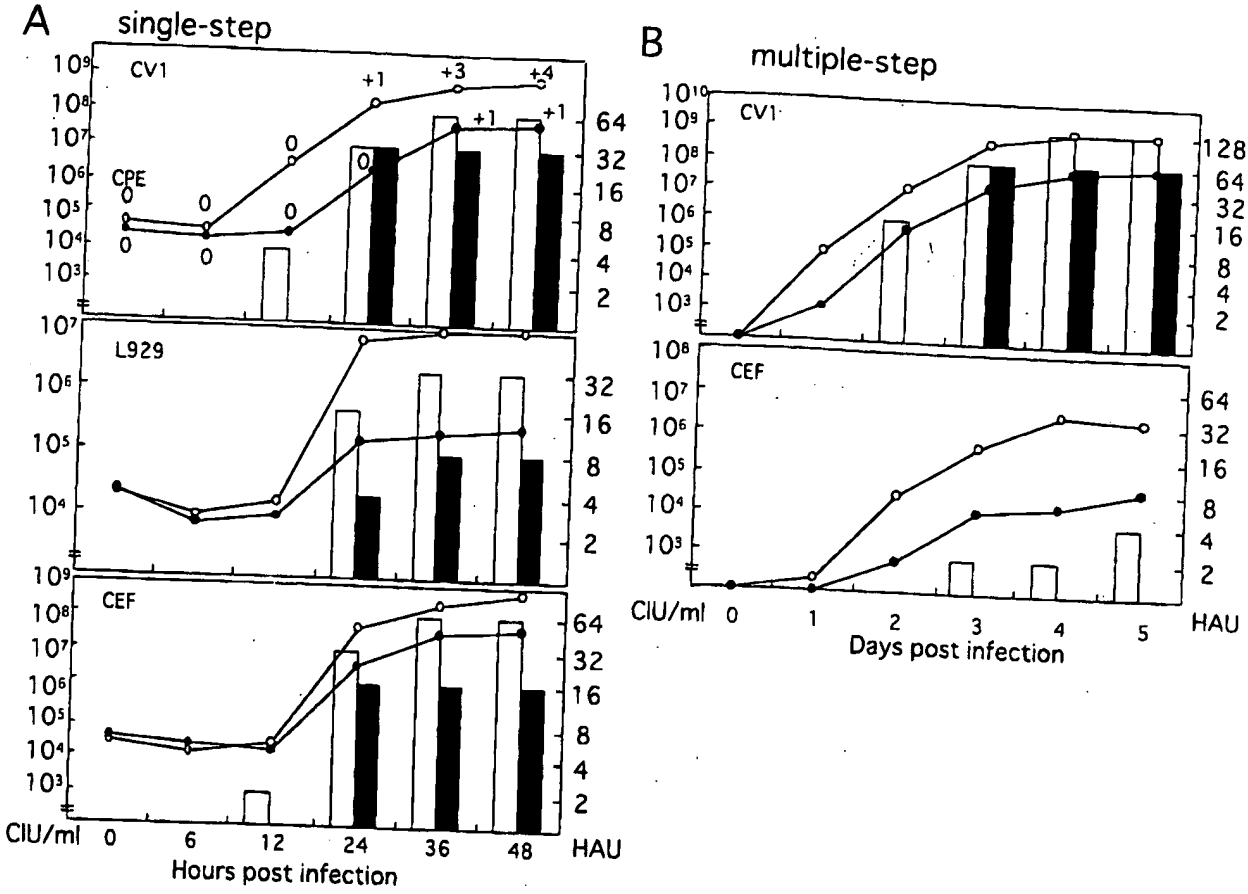

Figure 3 Growth of C/C′(−)a in various cells in culture. Cells were infected at a multiplicity 5.0 (A, single-step) or 0.001 (B, multiple-step) CIU/cell and infectivity (lines) and HAU (bars) were determined at various periods. Filled symbols or bars, C/C′(−)a; open symbols or bars, wild-type. The degree of the cytopathic effect on CV1 cells was graded on a scale of +1 to +4: rounding or lysis of nearly 100% (+4), 75% (+3), 50% (+2) and 10–25% (+1) of cells.

might not be absolutely indispensable for SeV replication in cells in culture but contributed greatly to maintaining a full replication capability. This requirement of C/C′ has so far appeared to be ubiquitous amongst cells of various species and tissues.

Transcription, translation and genome replication of the C/C′(−) mutants

The wild-type and C/C′(−)a viruses were compared by their gene expression in CV1 cells using Northern hybridization with N, P and M specific probes (Fig. 4A). Because of severe cytopathic changes, scored as +3, at 36-h post-infection (p.i.) in the wild-type infection (Fig. 3A), the comparison was made earlier, during the period up to 26 h p.i. Clearly, the N, P and M mRNAs were already at almost maximum levels after 12 h p.i. for the wild-type and remained so throughout the experiment. In marked contrast, their levels in the mutant-infected cells were much lower at 12 h and then gradually increased, becoming nearly comparable o those of the wild-type at 26 h (Fig. 4A). These different developmental patterns of viral transcripts appeared to be well correlated with those of the translation products studied by Western blotting. As shown in Fig. 4B, as all detectable viral proteins gradually increased with time but were consistently lower in the C/C′(−) virus-infected cells than the wild-type infection. SeV full length RNAs-are resolved in the 50 S position but at amounts much lower than the mRNAs. In addition, t .e aggregation of mRNAs sometimes yields nonspecific artifacts in this position. We therefore prefer semiquantitative RT-PCR analysis with specific probes to the simple Northern technique as a method to detect genome RNAs (Kato *et al.* 1997a). Here, we used the

A
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P —
M —
GAPDH
genome
anti-genome
B

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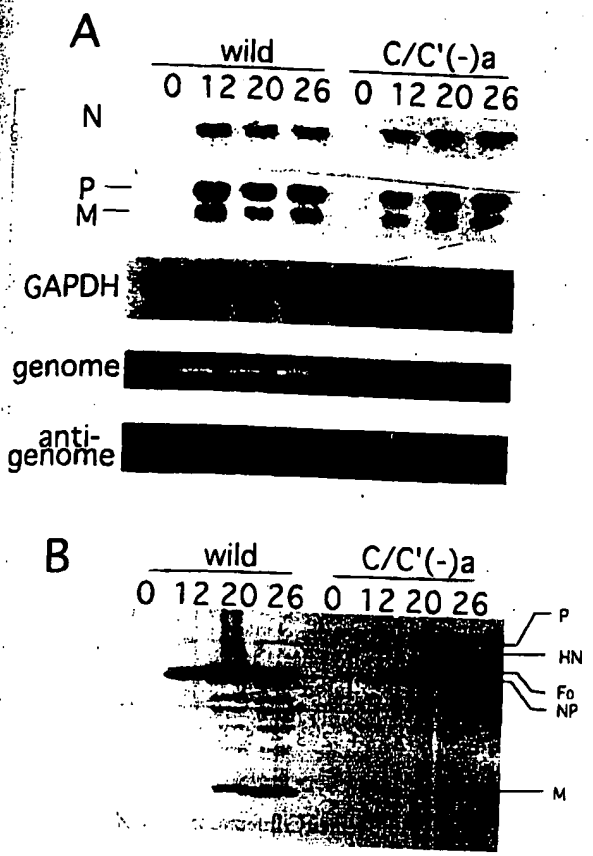

Figure 4 Transcription, translation and genome replication of the wild-type and C/C′(−)a viruses in CV1 cells. Total RNA was extracted from CV1 cells infected with each virus at a multiplicity of 5.0 CIU/cell at various hours indicated on the top of each lane, and analysed by Northern hybridization with probes specific for N, P and M genes (A). The same RNA samples were processed for RT-PCR to detect antigenome and genome RNA separately, with specific primers as described in Experimental procedures. In parallel, protein lysates were obtained from a companion set of infected cultures and analysed by Western blotting with anti-SeV polyclonal serum (B).

leader-N region and L-trailer region specific PCR primers to selectively detect the genomic and antigenomic RNAs, as described in Experimental procedures. As shown in Fig. 4A, the amounts of both strands were comparably reduced in the C/C′(−)a infected cells than in the wild-type infected cells up to 26 h p.i. Although the two different procedures (Northern hybridization and semiquantitative RT-PCR) did not allow for an exact comparison, the genome amplification appeared to be more severely impeded than the gene expression (Fig. 4A). Two other C/C′(−) viruses displayed very similar phenotypes to those of C/C′(−)a with respect to transcription, translation and genome replication (data not shown). These results strongly suggest that C and/or C′ proteins are required for full level SeV RNA synthesis. This requirement appeared to be stricter for the genome amplification than for the gene expression.

In vivo replication and pathogenicity of C/C′(−) viruses

SeV causes fatal pneumonia in the natural host, mice. To assess the contribution of C/C′ proteins to SeV *in vivo* pathogenicity, we examined the ability of C/C′(−) viruses to replicate and produce lesions in the target organ, the lung. The effect of infection on body weight was also examined because of its use as an indicator of SeV pathogenicity (Kiyotani *et al.* 1990). Figure 5 summarizes the results obtained following the intranasal inoculation of 5×10^6 CIU of the wild-type and the three C/C′(−) viruses. The wild-type SeV greatly disturbed body weight gain, and killed two of the three mice by day 7 p.i. The infected lungs manifested severe pathological changes, as indicated by high consolidation scores late in infection. In marked contrast, none of the three C/C′(−) viruses were lethal, affected body weight gain—except for a very slight disturbance at 1 day p.i., or produced lesions in the lung throughout. The C/C′(−) viruses were thus almost totally attenuated or virtually nonpathogenic. Their nonpathogenic nature was further substantiated by the almost complete lack of histological changes in the lungs throughout the infection.

The virus titres in each lung in each experimental group were determined. The titres immediately after inoculation (day 0) were very similar among the experimental groups, confirming that infections were initiated with a nearly identical input dose (Fig 5). The wild-type virus grew rapidly in the lung, reaching a peak titre of as high as $10^{8.5}$ CIU per lung on day 5 and then began to be gradually cleared. In sharp contrast, all three C/C′(−) were hardly able to grow, and were rapidly cleared out of the body, becoming undetectable by day 7. This virtual lack of replication capability seems to be well correlated with the above described nonpathogenic nature of the mutant viruses. There was a very slight increase in titre at 1 day p.i. with C/C′(−)b, maintenance of the initial titre for a day with C/C′(−)c and no such increase nor maintenance but an instantaneous clearance for C/C′(−)a. These small differences were not at all unique to each mutant, but represented fluctuations from experiment to experiment. Thus, all three mutants were thought to have the

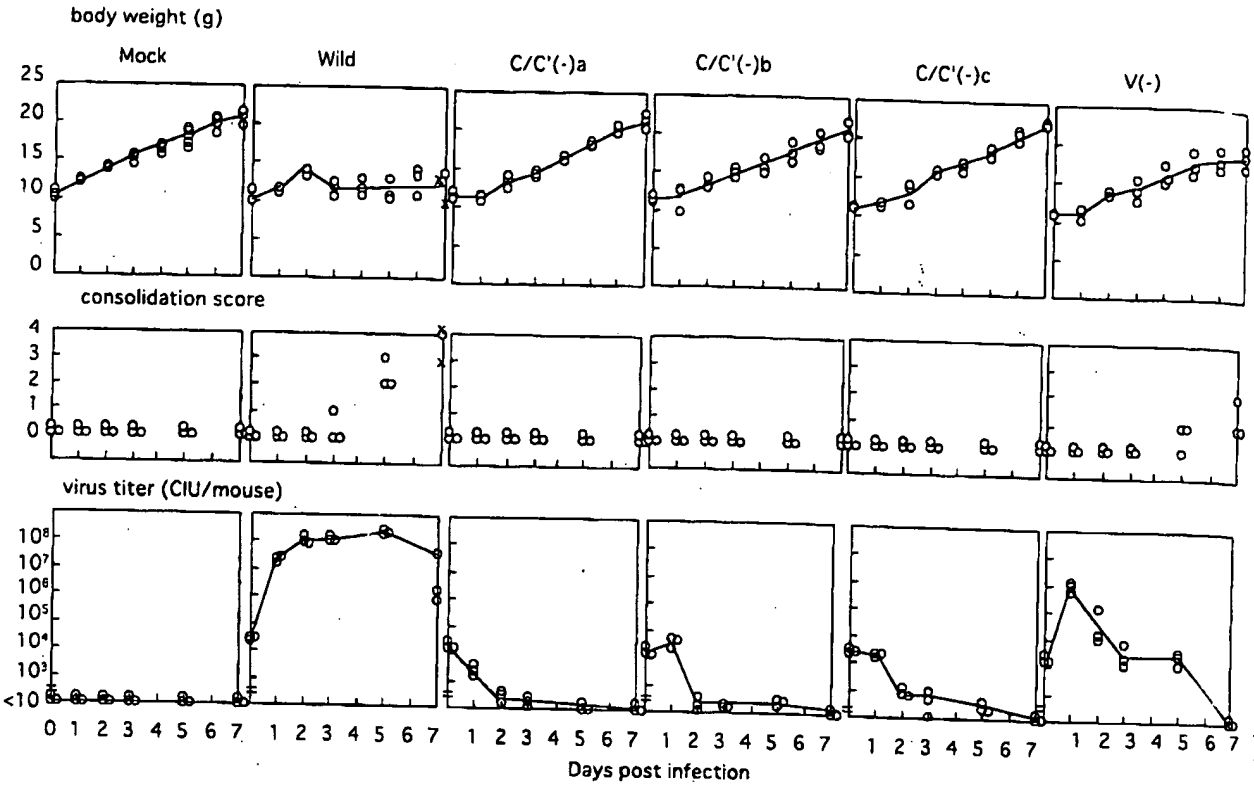

Figure 5 Replication of the wild-type, C/C′(−) and V(−) viruses in mice and their pathogenicity. Infections were initiated by the intranasal inoculation of 10^6 CIU per mouse. At appropriate time points, three mice were weighed (top), and consolidation scores (middle) and virus titres in the lungs (bottom) were determined. All these values are individually shown for each mouse. Dead mice are marked by X. Mock: mock infected with phosphate buffered saline.

potential to replicate equally, though only marginally, at the beginning of infection. This may be trivial in the face of the virtual lack of subsequent replication, but could be related to a slight impeding effect on body weight gain in the corresponding period, which was commonly found with the three mutants (Fig. 5). Again it should be noted that despite the above described amino acid changes unique to each virus, all three C/C′ viruses behaved very similarly *in vivo* and shared a very similar nonpathogenic phenotype.

We have previously shown that the deletion of another P gene product, the V protein, also resulted in strong attenuation *in vivo* (Kato *et al.* 1997a,b). Here, we made a direct comparison of V(−) and C/C′(−) viruses in order to evaluate the relative importance of V and C/C′ proteins for SeV pathogenesis. Exactly as described (Kato *et al.* 1997a), the V(−) virus replicated almost normally at least within the initial day of infection, reaching a comparable titre of about $10^{7.0}$ CIU per lung, though the virus was then rapidly cleared. Though strongly attenuated, the V(−) virus was still able to produce low scores in lung lesion and affected body weight slightly but significantly (Fig. 5). Thus, the deletion of C/C′ proteins was found to cause an even more profound attenuation than V deletion.

Silencing the expression of all four C proteins further attenuates the virus but is not lethal

A successful rescue of C/C′(−) viruses indicated no absolute requirement of C and C′ proteins for SeV replication. However, their replication capability both *in vitro* and *in vivo* were severely impaired (Figs 3–5). In addition, their rescue rates from cDNAs, as judged by the number of transfected cells and egg-passage required for virus recovery, were greatly reduced (Table 1). On the other hand, Y1 and Y2 frames were open in C/C′(−) viruses, which largely overlap C′ and C frames (Fig 1A). Moreover, Y1 and Y2 expression was greatly augmented in C/C′(−) viruses (Fig. 2). Therefore, the possibility was raised that Y1 and Y2 proteins

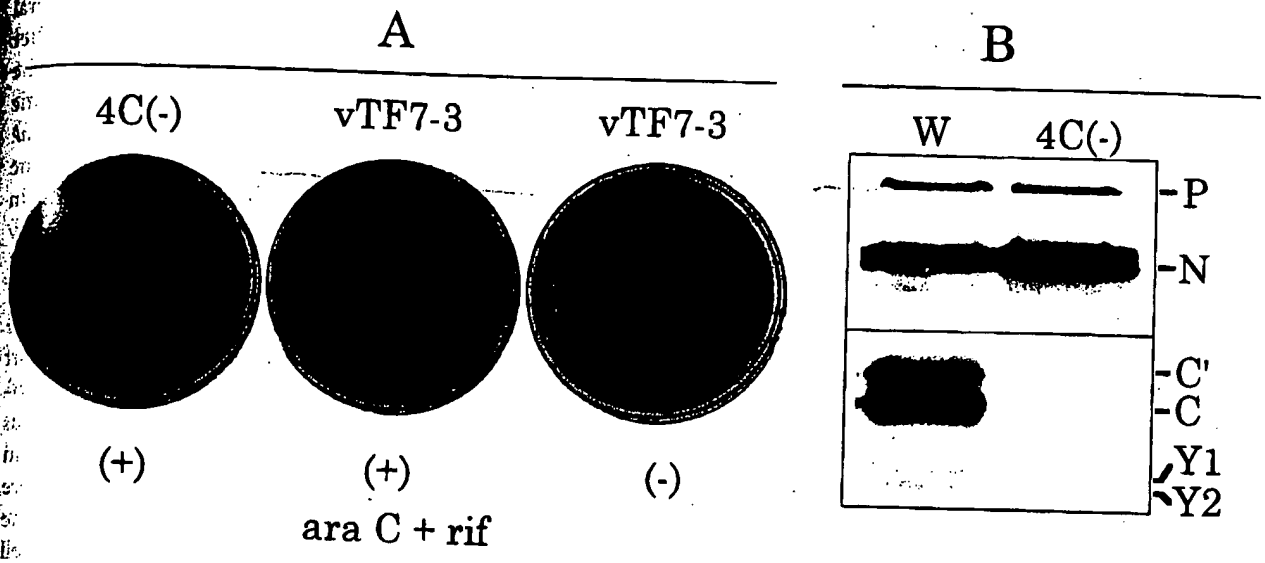

Figure 6 Plaques of 4C(−) virus (A) and knockout of all four C proteins in the virus(B). Plaque formation on CV1 monolayer cells was done in the presence (+) of ara C (40 μg/mL) and rifampicin (rif) (100 μg/mL), which completely inhibited the development of vTF7-3 plaques. The expression of C and other proteins from 4C(−) in CV1 cells was compared with that of the wild-type, by Western blotting as in Fig. 2, using anti C mono-specific and anti SeV polyvalent rabbit sera. Cell samples were harvested at 38 h p.i. following inoculation with undiluted 4C(−) virus stock or 10^{-5} diluted wild-type and incubation.

compensated for the loss of C and C′ proteins to confer to the C/C′(−) viruses some but not full replication capability. In other words, an important issue arose, that SeV C proteins may not merely be auxiliary but that at least one of them may be essential for SeV replication. To address this issue, we attempted to further silence the Y1 and Y2 expression from C/C′(−) virus by mutagenesis I + II (Fig. 1B). This was not easy, but starting with as many as 10^7 transfected cells and following three successive passages in eggs, we were eventually able to recover a virus named 4C(−) from one of the two mutant plasmids. The 4C(−) virus was, however, detectable only as extremely low PFU but not as HAU (Table 1). As vTF7-3 coexisted in the stock in nearly the same amounts (8.2×10^4 PFU/mL), 4C(−) virus plaques were selectively produced in the presence of ara C and rifampicin, which completely suppressed the development of vTF7-3 plaques (Fig. 6A). An additional passage slightly improved the virus yield (passage 4 in Table 1), while the vTF7-3 titre was fortunately reduced to 4.0×10^2 PFU/mL. We carefully diluted this virus preparation to an end-point, propagated it once again in eggs, and eventually obtained the 4C(−) stock (passage 5 in Table 1), from which the vTF7-3 vaccinia virus was completely eliminated (< 10 PFU/mL). The final titre of 7.0×10^6 PFU/mL was less than one-tenth of those of C/C′ viruses and was reduced by several logs, compared with that of the wild-type, indicating that the 4C(−) virus was critically impaired. This critically impaired replication capability of 4C(−) was confirmed in CV1 and other tissue culture cells tested (data not shown). With this stock virus, we confirmed a successful knockout of all four C proteins by Western blotting (Fig. 6B). We also confirmed the retention of the introduced mutations without accidental mutations in the P gene (data not shown). It should be noted that no such an accidental mutation as Glu (17) to Gly found for C/C′(−)a virus occurred in the 4C(−) virus. These results clearly demonstrated that SeV, which expresses none of the C proteins, is strongly impaired but viable, establishing for the first time that SeV C proteins fall in the category of a nonessential gene product. However, extremely low titres of 4C(−) virus have not enabled any pathogenicity studies to be carried out in mice.

Discussion

Our system for the recovery of infectious SeV from cDNA has opened the way to settling hitherto unsolvable issues regarding the accessory proteins of *Paramyxovirinae* (accessory in the sense that they are not expressed by all the members of this subfamiliy) in the context of not only viral replication *in vitro* but also

pathogenicity *in vivo*. It was first demonstrated that the SeV V protein encodes a luxury function required for maintaining a high viral load and causing fatal pneumonia in mice (Kato *et al.* 1997a,b). Here, C/C′ knockout was seen to render SeV almost totally incapable of replicating and virtually nonpathogenic in mice, indicating the indispensability of C/C′ proteins *in vivo*. The SeV C/C′ and V proteins therefore represent the accessory gene products whose absolute (C/C′) or partial (V) requirement for *in vivo* replication and pathogenesis was demonstrated for the first time for *Mononegavirales*.

Not absolute but strong dependency on the C/C′ proteins was demonstrated, even at a tissue culture level. The C proteins thus contrast sharply with the V protein which so far did not appear to be required at all, or whose knockout instead resulted in augmented gene expression and replication (Kato *et al.* 1997a; Yu *et al.* 1997). The 181-residue-long Y1 and 175-residue-long Y2 proteins are identical to the carboxyl-terminal portion of the 204-residue-long C and the 215-residue-long C′ proteins. Their preserved and even enhanced expression from C/C′(−) viruses might have compensated for the loss of C and C′ proteins. Indeed, this possibility has been suggested by a still more attenuated phenotype of 4C(−) virus in which Y1 and Y2 expression was further silenced. A more important message coming from the successful recovery of 4C(−) virus, was that SeV C proteins are categorically nonessential gene products, even though their contribution to viral replication capacity is quite great. Incapacity to recover an SeV in which all four C frames are silenced (Garcin *et al.* 1997; Tapparel *et al.* 1997) does not appear to preclude the possibility that the virus is present below detectable levels. Our success of 4C(−) creation could be owing to an extremely high efficiency of virus rescue from cDNA (Palese *et al.* 1996; Rose 1996).

The strong dependency on the C/C′ proteins may be unexpected in view of the absolute dispensability of measles virus and VSV C proteins in tissue culture replication (Radecke & Billeter 1996; Kretschmar *et al.* 1996). However, it remains to be elucidated whether the C proteins of these viruses have a function which is involved in a specialized role in *in vivo* infections. It has also to be noted that SeV C proteins exhibit little discernible sequence homology with the measles virus and VSV C proteins (13–17%), though they are highly homologous to those of hPIV1 (66.5%) and hPIV3 (38.4%). Whether essential or nonessential, C proteins can play a critical role which is specialized to the latter particular members of *Paramyxovirinae*.

SeV C proteins were previously found to inhibit viral mRNA synthesis (Curran *et al.* 1992). They further prevent the genome amplification templated by the negative strand genomic RNA but not the antigenomic RNA, thus in a promoter-specific fashion (Cadd *et al.* 1996). These down-regulations are reminiscent of the transcription inhibition associated with the protein encoded by the ORF2 in the M2 gene of respiratory syncytial virus (RSV) (Collins *et al.* 1996) and VSV M protein (Clinton *et al.* 1978). If exerted late in infection, moderation of RNA synthesis may be beneficial for the viruses in restricting excess cytopathogenicity to abort further progeny production and in rendering nucleocapsids quiescent prior to budding. These previous findings and views, however, do not appear to be in accord with our observations that C/C′(−) viruses were severely attenuated in gene expression and genome replication from the very beginning of infection as well as in cytopathic capacity and that 4C(−) virus was still more attenuated. Moreover, the co-infecting wild-type virus which should supply C proteins *in trans* was found to greatly enhance the RNA replication of 4C(−) virus (data not shown). Thus, our data has so far suggested some up-regulatory but not down-regulatory role in helping viral RNA synthesis and replication for SeV C proteins, which seems to be analogous to that of the ORF1-product of the RSV M2 gene (Collins *et al.* 1996). At the same time, our results do not preclude the possibility that an increase of replication selectivity caused by C proteins (Tapparel *et al.* 1997) is important for the SeV normal life cycle. A mini-replicon system suggested that SeV genome length should be a multiple of six nucleotides for efficient replication (Calain & Roux 1993). The stringency of this rule of six now appears to be greater in the presence of C proteins than in their absence (Tapparel *et al.* 1997). It is tempting to speculate that the C proteins contribute to the viral life cycle by preventing the replication of transcripts anomalous or aberrant in size, which could be generated in numerous rounds of copying in cells.

The V(−) virus grew normally in mice at least for the first day, although it was then rapidly cleared out of the body (Fig. 6; see also Kato *et al.* 1997a,b). This suggested that the V protein encodes a function required for SeV to evade innate immunity recruited at this time and thus presumably involving the induction of NK cells and interferons (Kato *et al.* 1997a,b). More recently, Garcin *et al.* (1998) reported similar phenotypes for a derivative of SeV with a point mutation in the C proteins and proposed a similar role for SeV C proteins. However, the virtual lack of multiplication of our C/C′ viruses in mice from the beginning of

Cell cultures and virus infecti n

Monkey kidney-derived cell lines LLCMK2 and CV1, mouse fibroblast line L929 and primary chick embryo fibroblasts were grown supplemented with 10% foetal bovine serum. Monolayer cultures of these cells were infected with the C/C′(−) viruses or the wild-type virus recovered from the parental cDNA at an input moi of 5 or 0.001 CIU/cell and maintained in serum-free MEM. In the latter experiment of multiple-step growth, trypsin (Gibco, 1:250) was added to the culture medium to give a final concentration of 5.0 μg/mL. At various hours(h) post-infection (p.i.), the culture media were assayed for CIU and HAU as described (Kiyotani *et al.* 1990). Plaque assays on CV1 cells were carried out with agar overlay medium containing the same amount of trypsin (Kato *et al.* 1996). If vTF7-3 coexisted, SeV specific plaques were produced by further adding ara C and rifampicin to the overlay medium.

Antisera and Western blotting

Anti-C and -P sera were raised in rabbits with the respective recombinant proteins (Kato *et al.* 1997a). Anti-V serum was raised in rabbits by immunizing specific synthetic oligopeptides exactly according to Curran *et al.* (1991). Anti-SeV rabbit polyclonal serum was described previously (Kato *et al.* 1996). For Western blotting with these sera, infected cell lysates were electrophoresed in 15% (for C protein) or 12.5% (for others) SDS-polyacrylamide gels (Laemmli 1970). The proteins in the gels were electrotransferred onto PVDF membranes (Millipore, Bedford) and probed with the above specific sera as described (Kato *et al.* 1995).

Northern hybridization and semiquantitative RT-PCR

Total RNA was extracted using RNAzol-B (Tel-Test Inc., Texas) from ≈ 10^6 CV1 cells infected with the wild-type SeV or C/C′(−) viruses at various time points p.i. The RNAs were ethanol precipitated, dissolved in formamide/formaldehyde solution, and then electrophoresed in 0.9% agarose-formamide/MOPS gels, and capillary transferred onto Hibond-N filters (Amersham, Buckinghamshire). They were hybridized with viral N gene-specific ($PstI^{571}$-RuI^{1760} fragment of pSeV(+)), P gene-specific ($SmaI^{2761}$-$SmaI^{3553}$ fragment) and M gene specific ($NdeI^{3700}$-$NdeI^{4578}$ fragment) probes that had been labelled with α-P^{32}-dCTP using the Multiprime DNA Labelling System (Amersham) (Kato *et al.* 1997a). The same filters were also hybridized with α-P^{32}-GTP labelled cellular gyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific riboprobe, synthesized *in vitro* using SP6 polymerase (Epicentre Technologies, Wisconsin). The semiquantitative reverse transcription (RT) and PCR-amplification to detect separately the genomic- and anti-genomic RNA fragments were performed exactly as described previously (Kato *et al.* 1997a) with two pairs of primers, pHvl (5′ ^{1}ACCAAACAAGAGAAAAAACA^{20} 3′) and pHvNPr1 (5′ ^{358}CC-ATGGCAAACAGCAAGACG^{339} 3′) which are specific for the leader-N region and pHvL1 (5′ ^{14907}TCT-AGAAGACTTGTGCTATC^{14926} 3′) and pHvt (5′ ^{15384}ACC/G ACAAGAGTTTAAGA-G^{15365} 3′) for the L-trailer region. The same RNA samples used for Northern hybridization were reverse-transcribed, either with pHvL1 primer for the genomic RNA detection or pHvNPr1 for the antigenomic RNA detection. The reverse-transcripts were then amplified for 15 cycles with the primer which was specific for each genomic or antigenomic strand. This procedure using 15 PCR cycles gave a linear correlation between the amounts of RNA template and PCR product (Kato *et al.* 1997a).

Infection of mice

Specific pathogen-free, 3-week-old male mice of ICR/Crj (CD-1) were purchased from Charles-River, Japan and used for the experimental virus infection. Eighteen mice were used for each experimental group for intranasal infection (10^6 CIU per mouse) with the mutant and wild-type viruses. At 0, 1, 2, 3, 5 and 7 days p.i., three mice from each experimental group were sacrificed, and consolidation scores and virus titres in the lungs were measured as previously described (Kiyotani *et al.* 1990; Kato *et al.* 1997a,b). Body weights were measured daily for three mice.

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at the fusion protein gene

Kato, Atsushi; Kiyotani, Katsuhiro; Hasan, Mohammad K.; Shioda, Tatsuo; Sakai, Yuko; Yoshida, Tetsuya; **Nagai, Yoshiyuki**
Department of Viral Infection, Institute of Medical Science, University of Tokyo, Tokyo, 108-8639, Japan

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Sendai Virus Gene Start Signals Are Not Equivalent in Reinitiation Capacity: Moderation at the Fusion Protein Gene

ATSUSHI KATO,[1,2] KATSUHIRO KIYOTANI,[3] MOHAMMAD K. HASAN,[1] TATSUO SHIODA,[4] YUKO SAKAI,[1] TETSUYA YOSHIDA,[3] AND YOSHIYUKI NAGAI[1,5]*

Department of Viral Infection[1] and Department of Infectious Diseases,[4] Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Department of Viral Diseases and Vaccine Control[2] and AIDS Research Center,[5] National Institute of Infectious Diseases, Tokyo 208-0011, and Department of Bacteriology, Hiroshima University School of Medicine, Hiroshima 734-8551,[3] Japan

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In paramyxovirus transcription, viral RNA polymerase synthesizes each monocistronic mRNA by recognizing the gene start (S) and end (E) signals flanking each gene. These signal sequences are well conserved in the virus family; nevertheless, they do exhibit some variations even within a virus species. In Sendai virus (SeV) Z strain, the E signals are identical for all six genes but there are four (N, P/M/HN, F, and L) different S signals with one or two nucleotide variations. The significance of these variations for in vitro and in vivo replication has been unknown. We addressed this issue by SeV reverse genetics. The luciferase gene was placed between the N and P gene so that recombinant SeVs expressed luciferase under the control of each of the four different S signals. The S signal for the F gene was found to drive a lower level of transcription than that of the other three, which exhibited comparable reinitiation capacities. The polar attenuation of SeV transcription thus appeared to be not linear but biphasic. Then, a mutant SeV whose F gene S signal was replaced with that used for the P, M, and HN genes was created, and its replication capability was examined. The mutant produced a larger amount of F protein and downstream gene-encoded proteins and replicated faster than wild-type SeV in cultured cells and in embryonated eggs. Compared with the wild type, the mutant virus also replicated faster in mice and was more virulent, requiring a dose 20 times lower to kill 50% of mice. On the other hand, the unique F start sequence as well as the other start sequences are perfectly conserved in all SeV isolates sequenced to date, including highly virulent fresh isolates as well as egg-adapted strains, with a virulence several magnitudes lower than that of the fresh isolates. This moderation of transcription at the F gene may therefore be relevant to viral fitness in nature.

Sendai virus (SeV) is an enveloped virus, with a nonsegmented negative-strand RNA genome of 15,384 bases, and belongs to the genus *Respirovirus* in the family *Paramyxoviridae*. The SeV genome is organized starting with the short 3′ leader region, followed by six genes encoding the N (nucleocapsid), P (phospho-), M (matrix), F (fusion), HN (hemagglutinin-neuraminidase), and L (large) proteins, and ending with a short 5′ trailer region. In addition to the P protein, the second gene expresses the accessory V and C proteins by a process known as cotranscriptional editing to insert a single nontemplated G residue (34, 35, 41, 42) and by alternative translational initiations, respectively (10, 28). The genome is tightly associated with the N protein, forming the helical ribonucleoprotein complex (RNP). This RNP, but not the naked RNA, is the template for both transcription and replication (30). There is only a single promoter at the 3′ end of the viral RNA polymerase, comprising the P and L proteins (11). By recognizing the short conserved end (E) and restart (S) signals at each gene boundary, the polymerase gives rise to leader RNA and each mRNA (7). There is a trinucleotide intergenic sequence between the E and S signals, which is not transcribed (9, 31). Since the reinitiation efficiency of transcription at each gene boundary is high but not perfect, the transcripts from the downstream genes are less abundant than those from the upstream genes. Therefore, each mRNA is not synthesized in equimolar quantities in infected cells but there is polar attenuation of transcription toward the 5′ end (7, 17, 30).

After translation of the mRNAs and accumulation of translation products, genome replication takes place. Here, the same viral RNA polymerase replicates the same RNP template, but now it somehow ignores the successive E and S signals for mRNAs and generates a full-length antigenomic positive-sense RNP (30). The polymerase enters the promoter at the 3′ end of the positive-sense RNP to generate the genomic negative-sense RNP, which serves as the template for the next round of transcription and replication.

The E sequence (3′-AUUCUUUUUU-5′ in the negative-sense genome) is completely conserved among the six genes in the SeV genome. Five U residues in the latter half allow polymerase slippage, generating poly(A). In contrast, the S signals are variable and are generalized as 3′-UCCCWVUUWC-5′ (9). They are UCCCACUUUC for the P, M, and HN genes, UCCCAgUUUC for the N gene, UCCCuaUUUC for the F gene, and UCCCACUUaC for the L gene. Identical differences are seen in all SeV strains sequenced to date, regardless of differences in isolation procedure, passage history, and virulence for mice, the natural host, suggesting that the variations are locus specific (Table 1). Thus, it has to be defined whether these variations have any significance for SeV replication and pathogenesis. To address this issue, we took advantage of SeV reverse genetics, which has been previously employed for identifying the gene functions and their contributions to viral pathogenesis (21, 22, 26, 32, 33, 37).

In this study, the firefly luciferase gene fused with the novel upstream E and S signals was inserted in the downstream

* Corresponding author. Mailing address: Department of Viral Infection, Institute of Medical Science, University of Tokyo, Shirokanedai 4-6-1, Minato-ku, Tokyo 108-8639, Japan. Phone: 81-3-5449-5285. Fax: 81-3-5449-5409. E-mail: ynagai@ims.u-tokyo.ac.jp.

TABLE 1. Gene start sequences of various SeV strains with database accession numbers

Strain	Passage and propagation host	Mouse virulence[a]	N UCCCAgUUUC	P UCCCACUUUC	M UCCCACUUUC	F UCCCuaUUUC	HN UCCCACUUUC	L UCCCACUUaC
Z	Egg	Low	M30202	M30202	M30202	M30202	M30202	M30202
Fushimi	Egg	Low	X17218	X17008	X53050	D00152	X56131	X58886
Enders	Egg	Low	X00583	X00583	X00584	X00585	X00586	X00587
Harris	Egg	Low	M29347	ND[b]	K02742	X02131	M12397	ND
F1-R	Cell line	Low	M30203	M30203	M30203	M30203	M30203	M30203
ts-F1	Cell line	Low	M30204	M30204	M30204	M30204	M30204	M30204
T5	Cell line	Low	M69040	M69040	M69040	M69040	M69040	M69040
Oh-MVC11	Cell line	Low	AB005796	AB005796	AB005796	AB005796	AB005796	AB005796
Oh-M1	Mouse	High	AB005795	AB005795	AB005795	AB005795	AB005795	AB005795
Hamamatsu	Mouse	High	ND	ND	D11446	D11446	X57213	ND

[a] Low, LD_{50} values higher than 10^4 PFU/mouse; high, those less than 10^2 PFU/mouse.
[b] ND, not determined.

noncoding region of the N gene. The S signals were changed exactly according to the four naturally occurring variations described above. In the recombinant viruses, the N mRNA transcription starts by its own S signal and terminates by the synthetic E signal within the inserted reporter sequence. Reporter gene expression, which was driven by each of the different S signals, was quantitated and compared each to the other. Thus, assessed reinitiation activity was remarkably lower for the natural S signal of the F gene than for those of the other three. Furthermore, when the natural S signal for the F gene was converted to that with a higher reinitiation activity, the recombinant virus was found to replicate faster, be more cytopathic in cell culture, and be more virulent for mice. SeV replication capability thus appeared to be moderated by a modification of the F gene S signal, and the significance of such moderation is discussed.

MATERIALS AND METHODS

Cell cultures and virus infection. The monkey kidney-derived cell lines LLCMK2 and CV1, were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum at 37°C. Monolayer cultures of these cells were infected with the mutant viruses recovered from cDNAs at an input multiplicity of infection (MOI) of 10 PFU/cell unless otherwise noted and maintained in serum-free MEM. The wild-type SeV (Z strain) recovered from the cDNA (20) was used as a control.

Creation of an insertion site after the N ORF. The plasmid pSeV(+) contained a cDNA copy of the full-length SeV antigenome (20) and was used as the starting material for plasmid construction. Eighteen nucleotides (gagggcccgcggc cgcga) containing the *Not*I restriction site were inserted between nucleotides 1698 and 1699 at the 3′ end of SeV genome which was located within the 5′ noncoding (in the negative sense) region of the N gene as shown in Fig. 1 (38). For the insertion, we used site-directed mutagenesis by a PCR-mediated overlap primer extension method (16) essentially according to our previous report (12). Briefly, two primers (NmF, 5′-gagggcccgcggccgcga^{1699}TACGAGGCTTCAAGG TACTT^{1718}-3′, and NmR, 5′-tcgcggccgcgggccctc^{1698}TGATCCTAGATTCCTCC TAC^{1670}-3′) with overlapping 18-nucleotide ends and two outer primers (OP1,

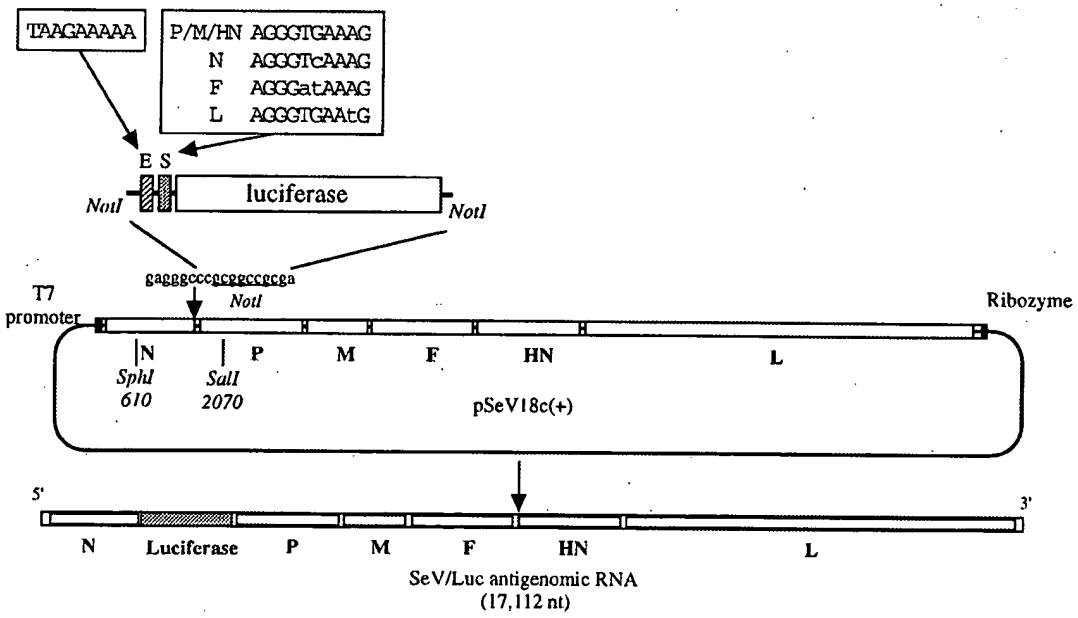

FIG. 1. Construction of plasmid pSeV18c(+) and insertion of the luciferase gene into the downstream region of the N ORF. An 18-nucleotide-fragment designed to contain a *Not*I site was inserted between nucleotides 1698 and 1699 from the 3′ end (38) of the SeV genome in pSeV(+) by site-directed mutagenesis. The resulting plasmid encoding the SeV antigenome with the 18-nucleotide insertion was named pSeV18c(+). The ORF of the luciferase gene was PCR amplified with four sets of *Not*I-tagged primers (ESn/NotLr, ESp/NotLr, ESf/NotLr, and ESl/NotLr) from the template plasmid, pHvLuc-RT4 (20), to generate the fragments containing the conserved E signal and each of the different natural S signals placed at the head of the luciferase gene. These amplified fragments were digested with *Not*I and introduced into the same site of pSeV18c(+). The resulting plasmids, named pSeV(+)SnLuc, pSeV(+)SpLuc, pSeV(+)SfLuc, and pSeV(+)SlLuc, were used to recover the recombinants SeV/SnLuc, SeV/SpLuc, SeV/SfLuc, and SeV/SlLuc, respectively.

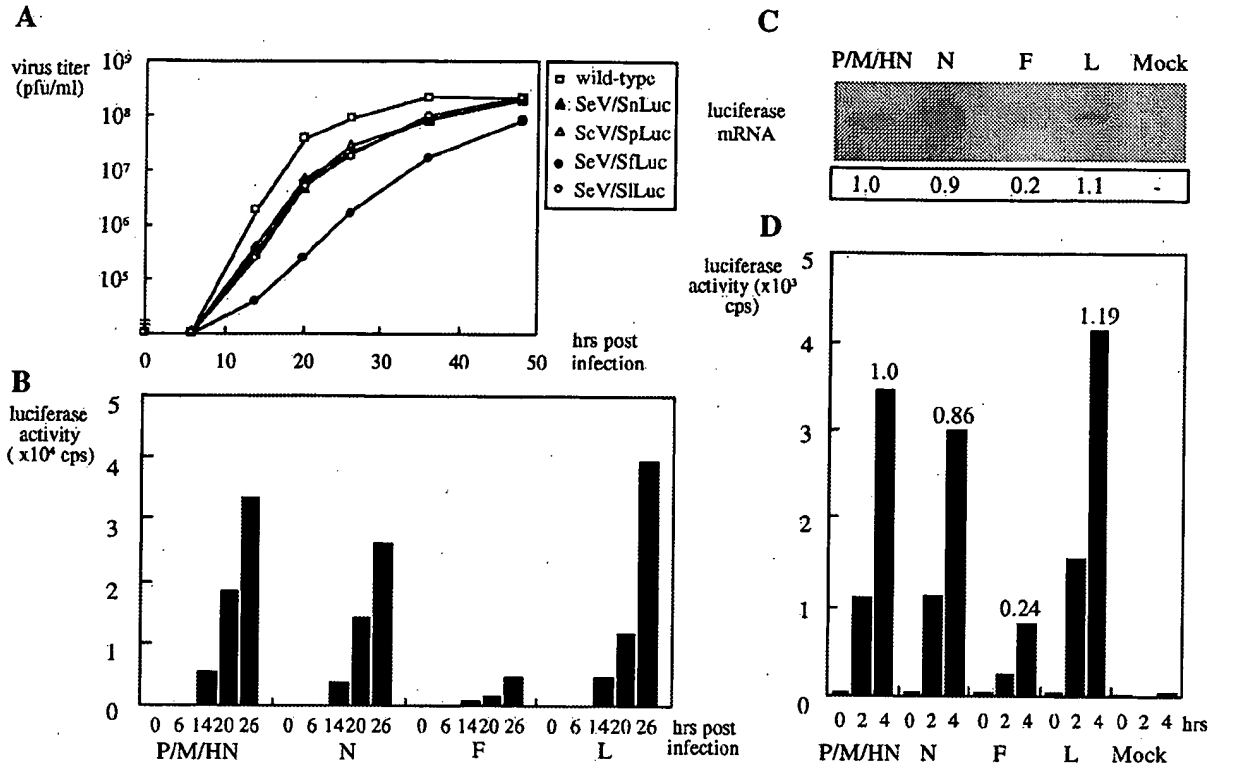

FIG. 2. Luciferase expression of SeV/SnLuc, SeV/SpLuc, SeV/SfLuc, and SeV/SlLuc. The recombinant viruses were inoculated onto CV1 cells at an MOI of 10 PFU/cell. Virus titers in the culture supernatants (A) and the luciferase activity in the cells (B) were measured at the times indicated. The recombinant viruses were inoculated onto CV1 cells at an MOI of 100 PFU/cell. The cells were cultured in the presence of cycloheximide for 12 h. Portions of the cells were harvested to prepare RNA and probed with the luciferase probe (C). The intensities relative to that of P/M/HN are also shown. The remainder of the cells was additionally incubated for 0, 2, and 4 h without cycloheximide to allow protein synthesis and lysed to measure the luciferase activity (D).

5′-^{61}CAAAGTATCCACCACCCTGAGGAGCAGGTTCCAGACCCTTTGCT TTGC^{105}-3′, and OP2, 5′-^{2467}TTAAGTTGGTVAGTGACTC^{2449}-3′) were synthesized. The first PCRs were performed with the OP1/NmF and the OP2/NmF primer pairs, using pSeV(+) as a template to gave rise to 1.6- and 0.8-kDa fragments, respectively. The second PCR was then performed with the OP1/OP2 primer pair, using the purified 1.6- and 0.8-kDa fragments to generate a single 2.4-kDa fragment with the 18 nucleotides. The 2.4-kDa fragment was purified and digested with *Sph*I and *Sal*I. Plasmid pSeV(+) was cut at positions 610 and 2070 on the SeV genome by these enzymes. The sequence of the resulting 1.47-kDa fragment was verified by sequencing on an AFLII automated DNA sequencer (Pharmacia, Uppsala, Sweden) and replaced with the same fragment of parental pSeV(+), thus generating pSeV18c(+), containing a unique restriction site after the N open reading frame (ORF) (Fig. 1).

Insertion of the luciferase gene with various S elements into pSeV18c(+). The luciferase gene from the firefly *Photinus pyralis* derived from pHVlucRT4(−) (20) was amplified by PCR with the following four primer pairs corresponding to the four different S sequences: ESp, 5′-TTgcggccgcGTAAGAAAAACTTAGG GTGAAAGTTCACTTCACGATGGAAGACGGCAAAAACAT-3′; ESn, 5′-T TgcggccgcGTAAGAAAAACTTAGGGTCAAAGTTCACTTCACGATGGAA GACGGCAAAAACAT-3′; ESf, 5′-TTgcggccgcGTAAGAAAAACTTAGGGA TAAAGTTCACTTCACGATGGAAGACGGCAAAAACAT-3′; and ESl, 5′-T TgcggccgcGTAAGAAAAACTTAGGGTGAATGTTCACTTCACGATGGAA GACGGCAAAAACAT-3′ and one common reverse primer NotLr, 5′-TCgcgg ccgcTATTACAATTTGGACTTTCCG-3′. Underlined are a new set of SeV E and S signals connected to the conserved intergenic trinucleotide; the lowercase letters represent the *Not*I restriction site. The boldface letters represent each unique nucleotide in the S signals. The 1.7-kDa fragments amplified with these primer pairs were purified, digested with *Not*I, and directly introduced into the *Not*I site of pSeV18c(+) (Fig. 1). The final constructs were named pSeV(+) SpLuc, pSeV(+)SnLuc, pSeV(+)SfLuc, and pSeV(+)SlLuc, respectively.

Mutagenesis to modify the S signal of the F gene in pSeV(+). A two-nucleotide exchange was performed on the S sequence of the F gene by two successive steps. At the first step, pSeV(+) was cleaved by *Ban*III at SeV positions 2088 and 5333 in pSeV(+) and the resulting 3.4-kDa fragment was recloned into the same restriction site of pBluescrit KS(+) (Stratagene, La Jolla, Calif.) to make pB/BanIII. Then, site-directed mutagenesis by a PCR-mediated overlap primer extension method (16) was performed as described above, using two inner primers (mGS1F, 5′-^{4810}CTTAGGGTGAAAGTCCCTTGT^{4830}-3′, and mGS1R, 5′-^{4830}ACAAGGGACTTTCACCCTAAG^{4810}-3′) and two outer primers (M1F, 5′-^{3931}TACCCATAGGTGTGGCCAAAT^{3951}-3′, and T7, 5′-TAATACGACTCA CTATAGGGC-3′). Underlined are the mutagenesis points. The first PCR was performed with the primer pairs MF1/mGS1R and T7/mGS1F, using pB/BanIII as a template, and yielded 0.9- and 0.6-kDa fragments, respectively. The second PCR was then performed with the M1F/T7 primer pair using these two purified fragments, generating a single 1.5-kDa fragment with the two nucleotide mutations. This fragment was purified and digested with *Ban*III and recloned into the same restriction site of pSeV(+) to make pSeV(+)mGSf. The authenticity of sequences to be cloned was verified by nucleotide sequencing.

Virus recovery from cDNAs. Viruses were recovered from cDNAs essentially according to the previously described procedures (20). Briefly, 2×10^6 LLCMK2 cells in 6-cm-diameter plates were infected with vaccinia virus (VV), vTF7-3, a gift of B. Moss (5), at an MOI of 2 PFU/cell. Then, 10 μg of the parental or mutated pSeV(+) and the plasmids encoding *trans*-acting proteins, pGEM-N (4 μg), pGEM-P or the mutated pGEM-P (see above) (2 μg), and pGEM-L (4 μg) were transfected simultaneously with the aid of Lipofectin reagent (DOTAP; Boehringer-Mannheim, Mannheim, Germany). The cells were maintained in serum-free MEM in the presence of 40 μg/ml of araC (1-β-D-arabinofuranosylcytosine) and 100 μg/ml of rifampin to minimize VV cytopathogenicity and thereby maximize the recovery rate (20). Forty hours after transfection, cells were harvested, disrupted by three cycles of freezing and thawing, and inoculated into 10-day-old embryonated hen eggs. After 3 days of incubation, the allantoic fluid was harvested. The titers of recovered viruses were expressed in hemagglutination units and PFU/milliliter as described previously (20). The helper VV contaminating the allantoic fluid of the eggs containing 10^8 to 10^9 PFU/ml of the recovered SeVs was eliminated by the second propagation in eggs at a dilution of 10^{-7}. This second passage of fluids, stored at −80°C, was used as the seed virus for all experiments.

Luciferase assay. The expression of luciferase activity from SeV was studied in 5×10^5 CV1 cells/well in 6-well plates at input multiplicities of from 1 to 300 PFU per cell. Under the single-cycle growth conditions, cells were harvested at 0, 6, 14, 20, and 26 h postinfection. Primary virus transcription was studied by incubating infected cells with 100 μg/ml of cycloheximide (Sigma, St. Louis, Mo.)

for 12 h, followed by incubation without cycloheximide for an additional 0, 2, and 4 h. The luciferase activity of harvested cells was measured by a luciferase assay kit (Promega, Madison, Wis.) with a luminometer (Luminos CT-9000D, Dialatron, Tokyo, Japan) as described before (12, 20).

RNA extraction and Northern hybridization. RNAs were extracted from the cells using TRIzol (Gibco BRL) or from culture supernatants and egg allantoic fluids using TRIzol/LS (Gibco BRL). For Northern hybridization, the RNAs were ethanol precipitated, dissolved in formamide-formaldehyde solution, electrophoresed in 0.9% agarose-formamide–MOPS gels, and capillary transferred onto Hibond-N filters (Amersham, Buckinghamshire, United Kingdom). They were probed with ^{32}P-labeled probes made by the multiprime labeling kit (Amersham). For the luciferase probe, the *NarI/HincII* (1,270-bp) fragment was purified from pHvlucRT4 (20). For the SeV N probe, the *PstI/PvuI* (1,189-bp) fragment was purified from pGEM-N. For the P probe, 792 bp of the *SmaI/SmaI* fragment was purified from pGEM-P. For the M, F, HN, and L probes, the *NdeI/NdeI* (878-bp), *BamHI/BamHI* (902-bp), *ScaI/ScaI* (1,108-bp), and *BamHI/BamHI* (1,654-bp) fragments, respectively, were purified from pSeV(+).

Western blotting. CV1 cells (2×10^5) grown in 6-well plates were infected at an MOI of 10 PFU per cell with the wild type or with SeV/mSf and harvested at various times postinfection. The cell pellets were lysed and run in sodium dodecyl sulfate–12.5% polyacrylamide gels (29) and immunoprobed with anti-SeV rabbit serum as described previously (19, 20).

Virus passages in eggs and virus detection. The wild-type SRV and SeV/mSf, which was a mutant virus recovered from the plasmid pSeV(+)mGSf, were coinoculated into two embryonated hen eggs with the respective doses of 10^4 PFU/egg or both 10^4 and 10^2 PFU/egg. Every 3 days postinoculation, the allantoic fluids were harvested and after dilution of 10^{-6}, reinoculated into new eggs. These reinoculations were successively repeated 10 times. The viruses grown in the allantoic fluids were semiquantitatively measured by reverse transcription-PCR (RT-PCR) with specific primer pairs. The RNA was extracted from 25 μl of each allantoic fluid, reverse transcribed with primer HvM (5′-^{4448}TTTTCTC ACTTGGGTTAATC^{4467}-3′) at 50°C for 30 min, using Superscript II (Gibco BRL), and heat denatured at 94°C for 2 min. The cDNAs were amplified with primers HvM and GS2WR (5′-^{4836}GCACTCACAAGGGACTTTCA^{4817}-3′) for wild-type SeV and with primers HvM and GS2MR (5′-^{4836}GCACTCACAAGG GACTTTat^{4817}-3′) for SeV/mSf as described previously (21, 28). The lowercase letters represent the mutated dinucleotides. The specific products were analyzed by electrophoresis in agarose gels as described above.

Infection of mice. Specific-pathogen-free, 3-week-old BALB/c and 4-week-old BALB/c (*nu/nu*) male mice were purchased from Charles River Laboratories. These mice were infected intranasally with 10^4, 10^5, 10^6, 10^7, or 10^8 PFU/mouse of the wild type or SeV/mSf under mild anesthetization with ether (23). Their body weights were individually measured every day up to 14 days. At 0, 1, 3, 5, 7, and 9 days postinfection, three mice in each group were sacrificed and the virus titers in the lungs were measured for BALB/c and BALB/c (*nu/nu*) mice inoculated with 10^4 PFU. Consolidation in the lungs was scored at the same time. The consolidation scores are expressed as follows: 0, no visible lesions or atrophy; 1, less than 25% of follicles affected; 2, 25 to 50% of follicles affected; 3, 50 to 75% of follicles affected; 4, more than 75% of follicles affected. When the mice died, one point was added for a score of 5.

RESULTS

Recovery of recombinant viruses expressing the luciferase gene under control of different S signals and their characterization. To insert the luciferase gene with a synthetic set of E and S signals, a unique *NotI* site was created downstream of the N ORF within the N gene essentially according to our previous work for the insertion of the same gene in the upstream noncoding region of the N ORF (12). In the present case, insertion of an 18-nucleotide (GAGGGCCCGCGGCCGCGA) stretch containing a *NotI* restriction site was not deleterious for virus rescue, with the recovery of a recombinant virus possessing full infectivity and replication capability similar to those recovered from the parental pSeV(+) (data not shown). Then, the luciferase genes fused to each of the four S sequences (Sn, Sp, Sf, and Sl) were inserted into the *NotI* site of the cDNA plasmid, pSeV18c(+) (Fig. 1). In all attempts, recombinant viruses were recovered. They were named SeV/SpLuc, SeV/SnLuc, SeV/SfLuc, and SeV/SlLuc, respectively, according to the S signals used.

The recombinant viruses were found to replicate more slowly than the wild type in CV1 cells (Fig. 2A), probably because of accommodation of an extra gene as long as 1,728 nucleotides (12). Among the four recombinants, SeV/SfLuc was still more attenuated because of reduced reinitiation activity at this particular S sequence (see below). Luciferase activities expressed from the recombinant SeVs were compared with each other. In all cases, the activities increased as infection proceeded (Fig. 2B) and their levels correlated well with those of luciferase mRNAs, which were identified as monocistronic transcripts by Northern hybridization (data not shown). These data unequivocally demonstrated that the synthetic E and S signals inserted just before the luciferase ORF were correctly recognized by the viral RNA polymerase. Remarkably, there were striking differences in luciferase activities, which appeared to be brought about by the S signal variations. The highest activity was obtained with SeV/SlLuc, and the lowest activity was obtained with SeV/SfLuc at 26 h postinfection (Fig. 2B). SeV/SpLuc and SeV/SnLuc had slightly lower activities than SeV/SlLuc at 26 h postinfection. However, this was not seen at 14 and 20 h postinfection. Thus, the reinitiation capacities of Sp, Sn, and Sl were regarded as comparable.

To see whether the above differences were primarily brought about at the level of transcription but not as part of the replication process, cells infected with the recombinants were incubated in the presence of cycloheximide, which inhibits protein synthesis and hence blocks viral replication requiring de novo viral protein synthesis. Under these conditions, only primary virus transcription catalyzed by the virion-associated RNA polymerase is allowed. After primary transcription and accumulation of viral mRNAs, cycloheximide was washed out from the culture. Cells were either lysed immediately to prepare the viral RNA (Fig. 2C) or incubated for an additional 0, 2, or 4 h to allow protein synthesis for measuring luciferase activities (Fig. 2D). The activities increased as the incubation period after cycloheximide removal was prolonged; SeV/SfLuc was again significantly lower than the other three in luciferase expression. The amounts of luciferase mRNA in each of the virus-infected cell groups correlated well with the activities of luciferase (Fig. 2C). The luciferase activities at 4 h of incubation were normalized to that of SeV/SpLuc, as this type of S signal is shared with three of the six genes. SeV/SnLuc and SeV/SlLuc activities were 0.86 and 1.19, respectively, and thus nearly comparable to SeV/SpLuc. In contrast, SeV/SfLuc reached only 0.24 of the control (Fig. 2D). These results strongly suggested that the signal used for F gene expression possessed a lower reinitiation potential than the other S signals.

Creation of an SeV mutant with an altered S signal for the F gene. The results described above suggested that there was a down-regulation of transcription at the F gene in the natural genome context of SeV. To substantiate this, we next created the mutant SeV/mSf, whose S signal for the F gene was replaced with that for the P/M/HN gene, and compared its replication with that of the wild type. SeV/mSf was found to grow faster than the wild type in CV1 cells (Fig. 3A). Cytopathogenicity, as manifested by cell rounding and cell detaching in the absence of trypsin and by cell fusion in the presence of exogenous trypsin to proteolytically activate the F glycoprotein, was greater for the mutant than for the wild type (Fig. 3B).

Expression of SeV/mSf genes. The mRNA levels in CV1 cells infected with the wild-type and mutant viruses were compared by Northern blotting at various times postinfection. As shown in Fig. 4A, the F and L transcripts from SeV/mSf were detected earlier and reached remarkably higher levels than those from the wild-type infection. The P and N transcripts were also detected earlier in SeV/mSf infection, although the peak levels were comparable. Accordingly, the levels of F_0 protein in the former were significantly higher than in the latter (Fig. 4B) at any time point throughout infection. The downstream gene products, HN and L, were not well resolved

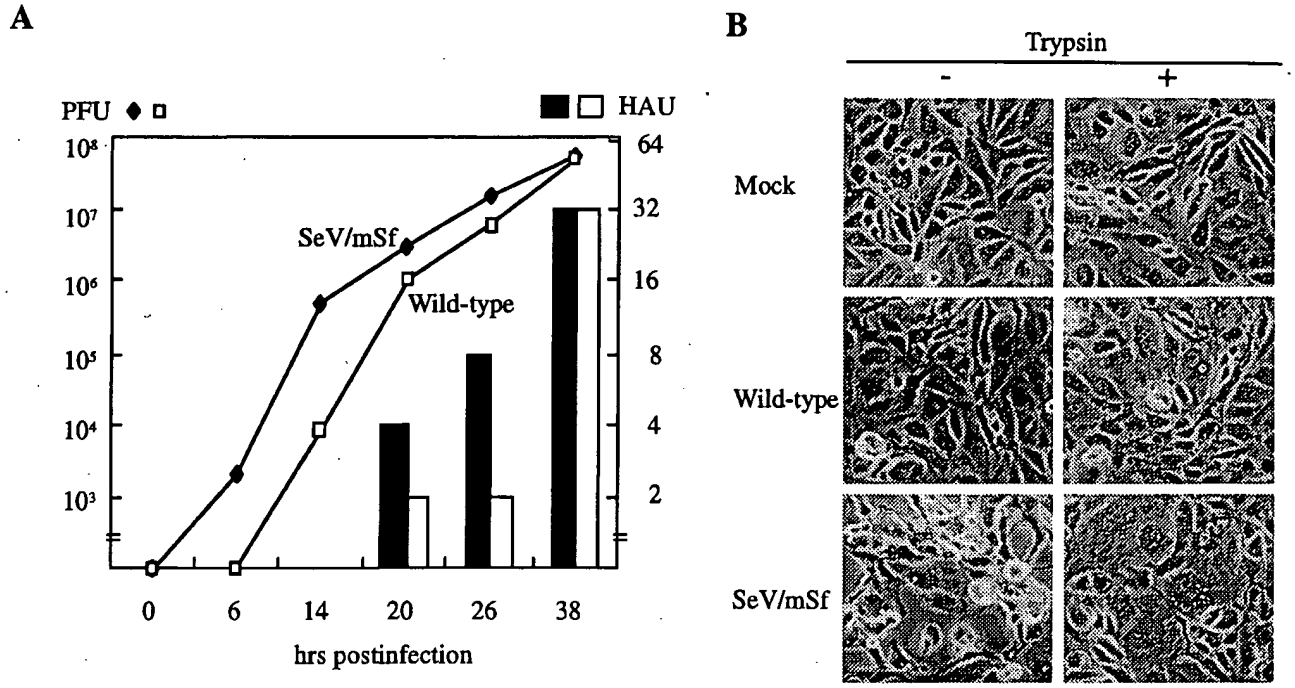

FIG. 3. Growth kinetics and cytopathogenicity of SeV/mSf. (A) The titers of the wild type and mutant SeV/mSf were measured at the times indicated, under single-cycle conditions. Open bars and filled bars represent hemagglutination units (HAU) of the wild type and mutant viruses, respectively. Lines with open and filled symbols represent PFU per milliliter of the wild-type and mutant viruses, respectively. (B) CV1 cells were infected with the wild type or SeV/mSf at an MOI of 20 PFU/cell in the presence (+) or absence (−) of trypsin. The pictures were taken at 48 h postinfection.

in this experiment. Enhanced expression of the F and L genes, but not of the N and P genes, was also clearly seen under the conditions of blocking de novo protein synthesis by cycloheximide (Fig. 4C). These results again unequivocally demonstrated that the S signal naturally occurring in F gene transcription possesses a lower reinitiation/promoter activity and hence down-regulates expression of F and downstream genes. Probably because of enhanced L gene expression, the virion RNA levels were higher for the mutant than for the wild type throughout infection (Fig. 4A). Earlier detection of mRNAs in mutant-infected cells, as demonstrated in Fig. 4A, might be also due to increased L gene expression.

Successive copassages of the wild type and SeV/mSf in embryonated hen eggs. Although wild-type SeV replicated more slowly than SeV/mSf in CV1 cells as shown in Fig. 2A, the possibility still remained that the naturally occurring down-regulation of transcription for the F and downstream genes would be advantageous for the persistence of SeV in the host, chicken embryos, than the artificially introduced up-regulation. We thus examined whether either the wild type or SeV/mSf would compete each other out during copassages of the two viruses in eggs. Coinfection was initiated with 10^4 PFU/egg of the two viruses or with 10^2 PFU/egg of the wild type and 10^4 PFU/egg of the mutant. At each inoculation up to the 10th passage, specific RT-PCR was performed to amplify either of the two viral genomes isolated from virions in the allantoic fluids, as shown in Fig. 5A. It was found that the wild-type genome was competed out by the eighth passage in the case of a 10^4-10^4 initial inoculation and by the fifth passage following a 10^2-10^6 initial inoculation (Fig. 5B). In control experiments, each virus was individually passaged and the genome sequences were determined. The results indicated that both viral genomes were stably maintained during 10 successive passages without any nucleotide change in the regions sequenced. These data indicated that the naturally occurring F gene S signal conferred no replication advantage on SeV at least in ovo.

Pathogenicity of wild-type SeV and SeV/mSf for mice. The final issue addressed in this work was whether the mutant SeV/mSf would replicate faster and be more pathogenic than the wild type in mice, the natural host, for which much more complex conditions exist than for cultured cells or eggs. Infections of BALB/c mice with the wild-type and mutant viruses were initiated intranasally at doses of 10^4, 10^5, 10^6, 10^7, and 10^8 PFU/mouse (Fig. 6). The mouse body weight gain was strongly disturbed by inoculations of 10^7 PFU of both viruses. All mice were killed by either virus at similar days postinfection. At 10^6 PFU, significant differences were found between the two viruses. Infection with SeV/mSf more strongly affected body weight gain than infection with the wild type. The former killed all mice, while the latter killed only one and allowed the remaining to regain the weight. At 10^5 PFU, all mice infected with the wild type showed a pattern of weight gain nearly comparable to that of mock-infected mice and survived, while those infected with the mutant did not. Thus, SeV/mSf was clearly more virulent than the wild type. The difference in virulence was quantitated by determining the 50% lethal dose (LD_{50}); the LD_{50} was 1.78×10^6 PFU for the wild type and 7.94×10^4 PFU for the mutant (Table 2). The mutant virus was thus 22 times more virulent than the wild type for the BALB/c strain. These results suggested that the naturally occurring F gene S signal attenuated SeV to some extent so that infected mice survive longer.

Cytotoxic T lymphocytes (CTL) modulate SeV pathogenesis in two different ways. They contribute to eliminating or clearing the virus from body on the one hand, and on the other, they accelerate disease progression by immunopathological pro-

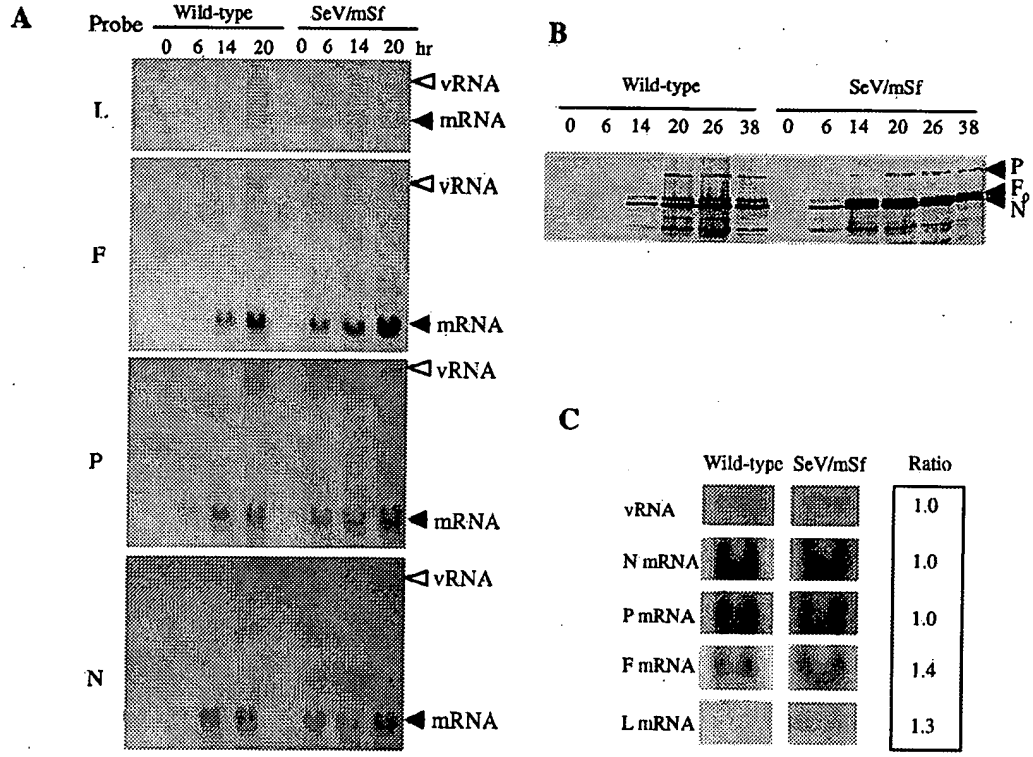

FIG. 4. Intracellular expression of viral genes. (A) CV1 cells infected with the wild type or SeV/mSf virus were analyzed by Northern hybridization with the viral N, P, F, or L gene probes at various times postinfection. The positions of mRNAs and genomic/antigenomic RNA (vRNA) are marked. (B) Intracellular expression of viral genes in CV1 cells was analyzed by Western blotting with anti-SeV antibody at the times (hours) indicated at the top of each lane. (C) CV1 cells were infected with either virus at an MOI of 100 PFU in the presence of cycloheximide. RNAs were extracted after 12 h of inoculation and analyzed by Northern hybridization. Approximate ratios of SeV/mSf to wild-type mRNA were determined with the BAS 2000 Image Analyzer.

cesses. We also examined the pathogenicity of the wild-type and mutant viruses for thymus-deficient nude mice. The LD_{50} values of each virus were comparable for nude mice and for the parental normal mice, and a similar difference (~40-fold) between the two viruses was found for the nude mice (Table 2). This result suggested that CTL and other thymus-dependent defenses did not play a major role in the pathogenesis of both wild-type and mutant viruses during the observation period (14 days), at least on the basis of LD_{50}. However, nude mice infected with both the wild-type and mutant viruses survived longer (Fig. 6). They nevertheless supported extended virus replication and increased lung consolidation (Fig. 7). These data suggested that CTL and other thymus-dependent responses played at least a part in viral pathogenesis (immunopathogenesis).

DISCUSSION

All nonsegmented negative-strand RNA genomes possess semiconserved, similarly sized S and E signals. Conservation of the sequences of these signals is extensively high within a viral genus and within a family and is extremely high among genes of a given virus species (4). However, there are some variations even within a virus species. In the case of the SeV S signal, there are four variations (9). Remarkably, these variations are fixed perfectly at the same respective genes of all strains, including fresh isolates, which are highly virulent for the natural host, rodents, and those isolated decades ago, passaged under different laboratory conditions and attenuated to various extents (Table 1). This fact suggested the possibility that the regulation of transcription, which presumably depends on the S motifs, is important for SeV life cycle and ecology.

Previously, several studies with model template systems of various nonsegmented negative-strand RNA viruses indicated that the S signals were indeed critically required for transcriptional initiation but able to tolerate variations in sequence to some extent (1, 2, 18, 25, 36, 40). Certain nucleotide exchanges in their S signals were shown to initiate less transcription, suggesting that gene expression was also modulated by naturally occurring variations in the viral life cycle (26, 27, 39). However, in the model template systems, any event required early in the natural life cycle, like primary transcription, is bypassed by the successive and constant supply of *trans*-acting proteins (32). The transcription and replication of minigenomes are uncoupled in these systems. T7 polymerase-expressing VV often used to produce the *trans*-acting proteins might mask the subtle effects of mutations by, for example, the post-transcriptional modifications by VV encoding capping enzyme. In addition, the transfection efficiencies might not be equal throughout the experiment. Thus, to address the roles of S and E signals comprehensively, it has been necessary to introduce relevant mutations into a full-length viral genome (3, 15).

The results obtained here by SeV reverse genetics clearly showed that the S sequence for the F gene was remarkably less potent in initiation than the other three S sequences. That the reduced luciferase gene expression by the F-specific signal was indeed caused primarily at the transcriptional level but was not a secondary result of replication was confirmed by blocking de

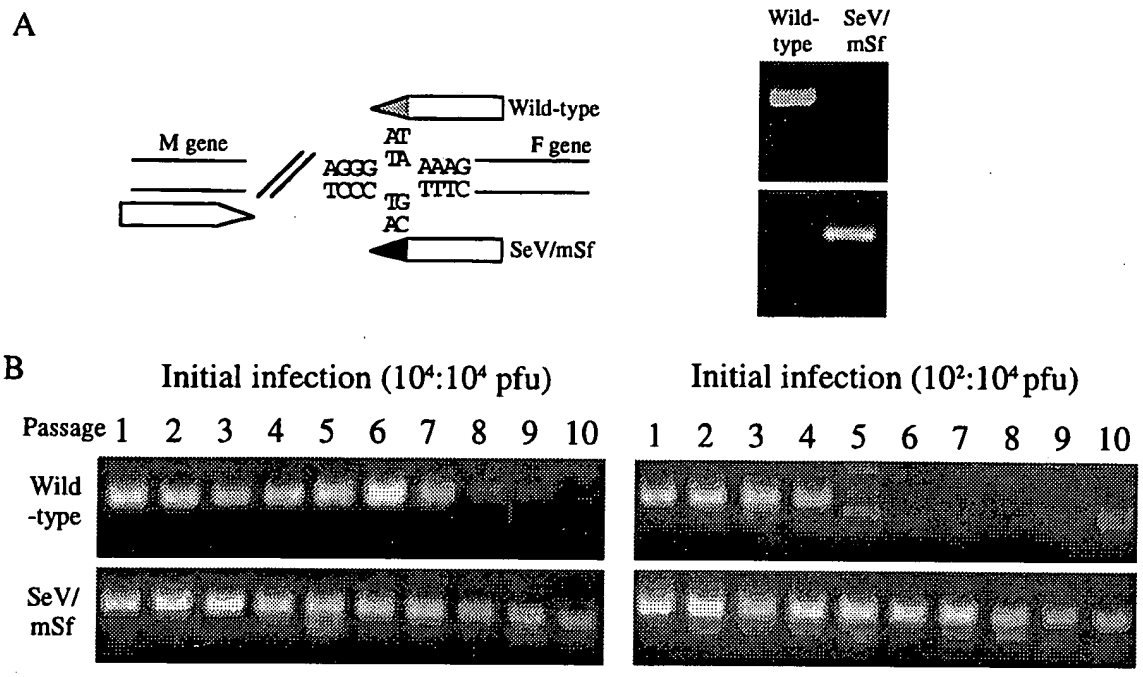

FIG. 5. Competition assays of the wild type and SeV/mSf in serial copassages. (A) Specific primer sets (left) detect either of the viral RNAs (right). (B) Each passage was initiated with input doses of 10^4 (SeV/mSf) and 10^4 (wild-type virus) PFU/egg or 10^4 (SeV/mSf) and 10^2 (wild-type virus) PFU/egg. The allantoic fluids were harvested every 3 days, diluted to 10^{-6}, and coinoculated into new eggs serially up to 10 passages. Viral RNAs were extracted and analyzed by a one-step RT-PCR method, using the specific primer sets.

novo protein synthesis and hence eliminating genome replication (Fig. 2). The primary transcription experiment further assessed that the reinitiation activity driven by the S sequence for the F gene was approximately one fourth those of the other three. These observations are in good agreement with the previous observation that the amounts of N, P, and M mRNAs in SeV-infected cells were almost equally high, but F and HN mRNAs were present in about threefold-lower amounts (17). The results taken together may argue against the view of simple polar attenuation of transcription for SeV, which is essentially linear toward the 5′ end of template. Rather, the transcriptional attenuation between the N and P genes as well as that between the P and M genes may be small or negligible. Although the main issue was the steep transcriptional attenuation at the subsequent F gene, the possibility that the low level of mRNA might be the result of the shorter life of the transcripts due to the differences at the S signal could not be ruled out. There is no plausible explanation for the extremely low copy numbers of L mRNA (about 1/30 those of N, P, and M mRNAs) (17). The reinitiation activity of the S sequence for the L gene studied by recombinant simian virus 5 with the reporter green fluorescent protein gene was considerably low (15). The same signal for respiratory syncytial virus (RSV), studied by a model template system, appeared to be as active as the other RSV signals (27). The same was true for SeV as shown here. The contribution of the S signal to L gene expression thus appeared to be variable among the viruses, or the copy number of the extremely long L gene transcripts may be influenced by some other factors, such as processivity of the polymerase.

The reinitiation capacity of different S sequences was then assessed by replacing the natural S sequence of the F gene with that of the P/M/HN genes and by examining the replication capability of the recovered virus (SeV/mSf) in cultured cells, in ovo, and in mice. It was unequivocally demonstrated that the inserted S sequence enhanced F and downstream gene expression, again at the transcriptional level (Fig. 4). The mutant virus with the new S sequence for the F gene replicated faster and was more cytopathic in cultured cells (Fig. 3) and competed out the wild-type virus in eggs (Fig. 5). Thus, the naturally occurring S signal for the F gene appears to moderate the initiation of F gene transcription and viral replication. Since polymerase enters the 3′ end of the genome in transcription as well as in replication, once moderated at the F gene, transcription of the downstream genes including the L gene encoding the catalytic subunit of polymerase was also moderated. Then, the next round of both replication and transcription could be affected, ultimately leading to reduced virion RNA synthesis and progeny production.

The in vivo study showed that SeV/mSf had 20 times lower LD_{50} values and hence was more virulent than the wild type for BALB/c mice (Table 2). CTL generally play a major role in virus clearance by eliminating virus-infected cells. They are also important for immunopathological processes leading to damage of infected cells and tissues. The increased level of F protein expression from the mutant virus would lead to a stronger CTL response. These aspects of CTL appeared to be involved not only in wild-type SeV pathogenesis but also in increased pathogenicity of SeV/mSf as determined by LD_{50}. Thus, the increased pathogenicity of SeV/mSf may simply be attributable to its increased replication capacity.

So far, our data suggest that the F gene S signal imposes a restriction of productive infection on SeV. Its presence in the natural SeV genome did not appear to be advantageous at least in ovo in competition assays with a signal with a higher promoter capacity (Fig. 5). It is therefore quite difficult to conceptualize the basis for the strong conservation of an S sequence with a lower initiation activity at the same position in

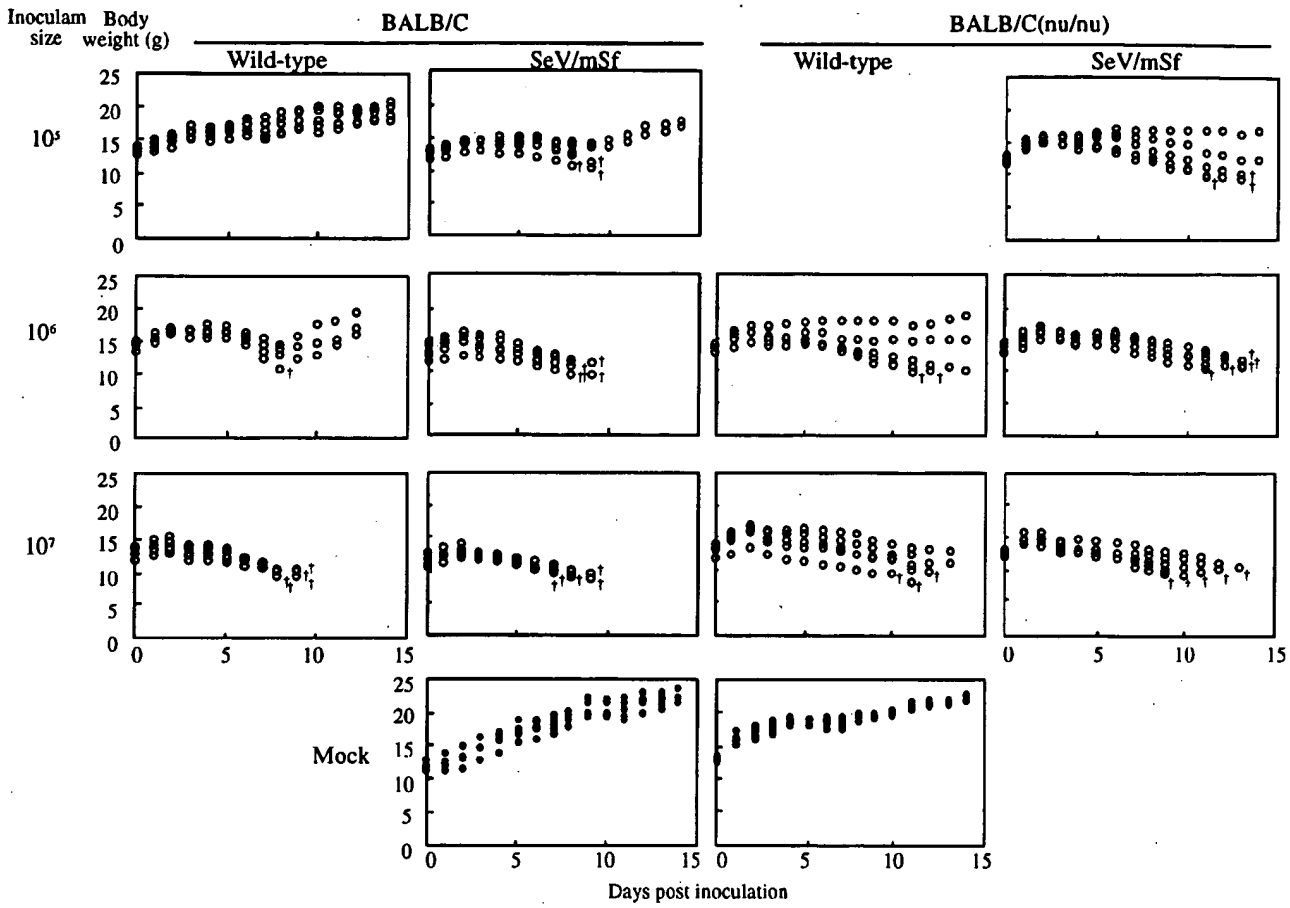

FIG. 6. Body weight gain of normal BALB/c and thymus-deficient BALB/c (*nu/nu*) mice infected with the wild-type and SeV/mSf viruses. Five mice were inoculated intranasally with various doses of virus (10^4 to 10^7 PFU per mouse). The weight gain of mice was measured in grams (top) every day up to 14 days postinoculation. Dead mice are marked by †.

SeV genomes sequenced to date, including those of highly virulent fresh isolates. One estimate suggested that the LD_{50} of a fresh isolate is as low as 10 PFU/mouse, whereas that of the egg-adapted strain used here was as high as 10^5 to 10^6 PFU/mouse (21, 22, 28). If such a virulent field strain is further potentiated by incorporating an S signal with a higher reinitiation activity into the F gene, infected rodents would be killed so rapidly that they would have less opportunity to transmit virus to new hosts. Indeed, it was recently shown that some newly emerging influenza virus isolates of the H5N1 subtype were too virulent to be transmitted to a neighbor host in a mouse colony (6, 8). Moderation of replication by transcriptional attenuation at the F gene may thus be advantageous for SeV to persist in nature.

TABLE 2. LD_{50} values of the wild-type and mutant viruses for BALB/c and nude mice

Virus and inoculum	BALB/c[a]	LD_{50}	BALB/c (*nu/nu*)[a]	LD_{50}
Wild-type				
10^8	5/5		NT[b]	
10^7	5/5		3/5	
10^6	1/4	1.78×10^6	2/5	3.16×10^6
10^5	0/5		NT	
10^4	0/5		0/5	
SeVmSf				
10^8	5/5		NT	
10^7	5/5		5/5	
10^6	5/5	7.94×10^4	5/5	7.94×10^4
10^5	3/5		3/5	
10^4	0/5		0/5	

[a] No. dead/no. inoculated.
[b] NT, not tested.

RNA-dependent RNA polymerase is error prone (13, 24). The natural S signal for the F gene was here shown to be less advantageous than that with a higher reinitiation activity in hen eggs. Thus, one can predict that SeV with an F start of higher reinitiation capacity would evolve during serial passages in eggs. However, this has not happened over the years, as indicated by strict conservation of the natural F start sequence in all strains sequenced to date. A similar situation is seen for another SeV *cis*-acting element, the editing motif in the P gene, as it is totally dispensable or even restrictive for viral replication under laboratory conditions but strictly conserved in SeV isolates, including egg-adapted strains (14, 22). These sequence conservations may be somehow required for optimizing replication capacity even in cultured cells and eggs. Alternatively, nucleotide misincorporation may not be equal throughout the genome but is somehow restricted in these regions. Based on this hypothesis, S signal mutation that down-

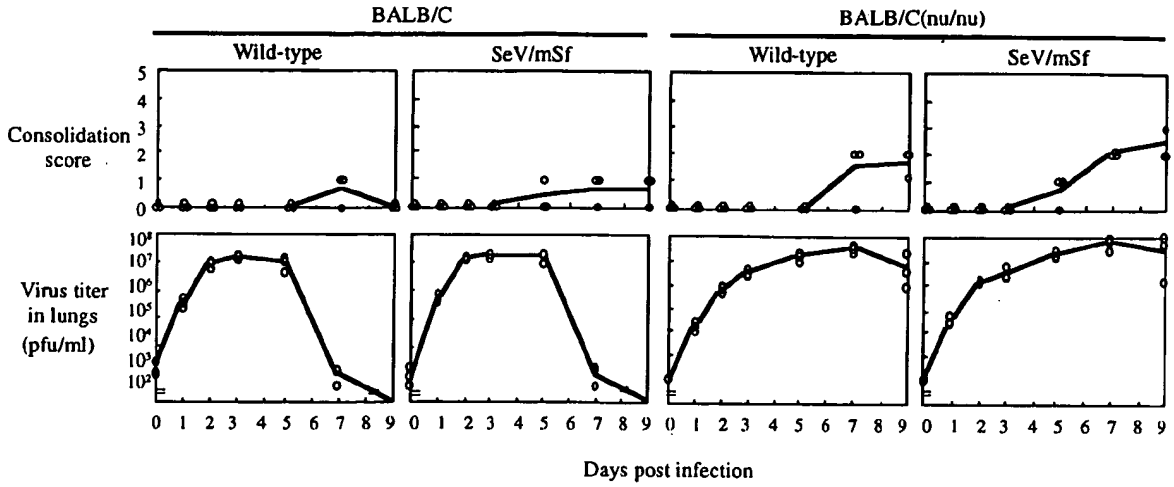

FIG. 7. Consolidation and viral loads in the lungs of BALB/c and BALB/c (*nu/nu*) mice. Each mouse was intranasally inoculated with 10^4 PFU of the indicated viruses. Three mice were sacrificed at 0, 1, 2, 3, 5, 7, and 9 days postinoculation to grade consolidation scores (top) and to determine virus titers in the lungs (bottom). All these values are individually shown for each mouse.

regulates F expression could be beneficial for SeV to persist in nature, since it was fixed early in the evolutionary process and then has been maintained in laboratory strains.

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generates antibodies that specifically react with RSV F protein, the first and second DNA sequences being under the transcriptional control of a promoter is described. Such **vector** may be used to produce an RNA transcript which may be used to immunize a host, including a human host, to protect the host against disease caused by **paramyxovirus**, particularly respiratory syncytial virus, by administration to the host.

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A Cytoplasmic RNA Vector Derived from Nontransmissible Sendai Virus with Efficient Gene Transfer and Expression

HAI-OU LI,[1] YA-FENG ZHU,[1] MAKOTO ASAKAWA,[1] HIDEKAZU KUMA,[1] TAKAHIRO HIRATA,[1] YASUJI UEDA,[1] YUN-SIK LEE,[1] MASAYUKI FUKUMURA,[1] AKIHIRO IIDA,[1]* ATSUSHI KATO,[2]† YOSHIYUKI NAGAI,[2]‡ AND MAMORU HASEGAWA[1]

DNAVEC Research Inc., Tsukuba-shi, Ibaraki 305-0856,[1] and Department of Viral Infection, Institute of Medical Sciences, University of Tokyo, Minato-ku, Tokyo 108-8639,[2] Japan

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We have recovered a virion from defective cDNA of *Sendai virus* (SeV) that is capable of self-replication but incapable of transmissible-virion production. This virion delivers and expresses foreign genes in infected cells, and this is the first report of a gene expression vector derived from a defective viral genome of the *Paramyxoviridae*. First, functional ribonucleoprotein complexes (RNPs) were recovered from SeV cloned cDNA defective in the F (envelope fusion protein) gene, in the presence of plasmids expressing nucleocapsid protein and viral RNA polymerase. Then the RNPs were transfected to the cells inducibly expressing F protein. Virion-like particles thus obtained had a titer of 0.5×10^8 to 1.0×10^8 cell infectious units/ml and contained F-defective RNA genome. This defective vector amplified specifically in an F-expressing packaging cell line in a trypsin-dependent manner but did not spread to F-nonexpressing cells. This vector infected and expressed an enhanced green fluorescent protein reporter gene in various types of animal and human cells, including nondividing cells, with high efficiency. These results suggest that this vector has great potential for use in human gene therapy and vaccine delivery systems.

Sendai virus (SeV) is an enveloped virus with a nonsegmented negative-strand RNA genome of 15,384 nucleotides and is a member of the family *Paramyxoviridae*. The SeV genome contains six major genes, which are lined up in tandem on a single negative-strand RNA. Three virus-derived proteins, the nucleoprotein (NP), phosphoprotein (P), and large protein (L; the catalytic subunit of the polymerase) form a ribonucleoprotein complex (RNP) with the SeV genomic RNA, and the RNP acts as a template for transcription and replication. Matrix protein (M) engages in the assembly of viral particles. Two envelope glycoproteins, hemagglutinin-neuraminidase (HN) and fusion protein (F), mediate the attachment of virions and penetration of RNPs into infected cells. F protein is synthesized as an inactive precursor protein F_0 and split into F_1 and F_2 by proteolytic cleavage of a trypsin-like enzyme. SeV replication is independent of nuclear functions and does not have a DNA phase. Therefore, it does not transform cells by integrating its genetic information into the cellular genome (16).

Methods to rescue infectious viruses entirely from cloned cDNA have been established for segmented and nonsegmented negative-strand RNA viruses (6, 22, 23, 26). Such reverse genetics technology has enabled the construction of genetically engineered viruses which carry additional foreign genes and opened the way for the development of gene transfer vectors from RNA viruses of this type (24). The vectors prepared by this method have shown a high efficiency of gene transfer and expression of foreign proteins in vitro (3, 12, 18, 21, 28, 32, 36). However, the recombinant paramyxoviruses constructed to date have contained all the viral structural genes and thus are replication competent, giving rise to fully infectious progeny capable of spreading in the body.

Here we report the development of a novel SeV vector that is capable of self-replication but incapable of infecting neighboring cells. The vector does not encode F protein, which is one of the endogenous envelope proteins, but instead incorporates it expressed in *trans*. We further show that an inserted enhanced green fluorescent protein (EGFP) reporter gene is vigorously expressed from this SeV vector in cells of various origins in culture, including human smooth muscle cells, hepatocytes, and lung microvascular endothelial cells, in primary cultures of rat cerebral cortex cells, and in the lateral ventricles and hippocampus of the rat brain. Thus, this F-defective vector appears to represent the important first step toward human gene therapy and vaccine delivery using SeV replicons.

MATERIALS AND METHODS

Virus. The attenuated SeV Z strain was used as a basis for the genome used in this study. Recombinant vaccinia virus vTF7-3 (9) expressing T7 RNA polymerase which had been inactivated with psoralen and long-wave UV light (34) was used for RNP recovery experiments. Recombinant adenovirus AxCANCre (14) expressing Cre recombinase was used for induction of F protein from LLC-MK_2/F7 cells.

Cell culture. A rhesus monkey kidney cell line, LLC-MK_2, was cultured in minimal essential medium (MEM) (Gibco-BRL, Rockville, Md.) supplemented with 10% heat-inactivated fetal calf serum (FCS). For virus propagation, LLC-MK_2/F7 cells were cultured in MEM containing cytosine arabinoside (araC) (Sigma, St. Louis, Mo.) at 40 μg/ml and trypsin (Gibco-BRL) at 7.5 μg/ml. Normal human smooth muscle cells, normal human hepatocyte cells, and normal human lung microvascular endothelial cells (Cell Systems Corp., Kirkland, Wash.) were cultured in SFM CS-C medium (Cell Systems Corp.). All cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.

Plasmid construction. To replace the F gene of SeV cDNA clone with the EGFP reporter gene, the 6.0-kb *Sac*I fragment of pSeV18$^+$b(+) (12) which contained the F gene was cloned into pUC18 (Stratagene, La Jolla, Calif.) to generate pUC18/Sac. A 1,698-bp fragment of the total open reading frame of the F gene in pUC18/Sac was deleted by a combination of PCR and ligation. For an upstream fragment of the F gene, the primer pair FF-1 (5′-GTTGAGTACTG CAAGAGC-3′) and FR-1 (5′-TTTGCCGGCATGCATGTTTCCCAAGGGGA GAGTTTTGCAACC-3′) was used, and for a downstream fragment, the primer pair FF-2 (5′-AAAATGCATGCCGGCAGATGATCACGACCATTATCAGA TGTCTTG-3′) and FR-2 (5′-CTAAAGTACCGCGCGACC-3′) was used (see

* Corresponding author. Mailing address: DNAVEC Research Inc., 1-25-11 Kannondai, Tsukuba-shi, Ibaraki 305-0856, Japan. Phone: 81-298-38-0540. Fax: 81-298-39-1123. E-mail: iida@dnavec.co.jp.

† Present address: Department of Viral Diseases and Vaccine Control, National Institute of Infectious Diseases, Tokyo 208-0011, Japan.

‡ Present address: AIDS Research Center, National Institute of Infectious Diseases, Tokyo 162-8640, Japan.

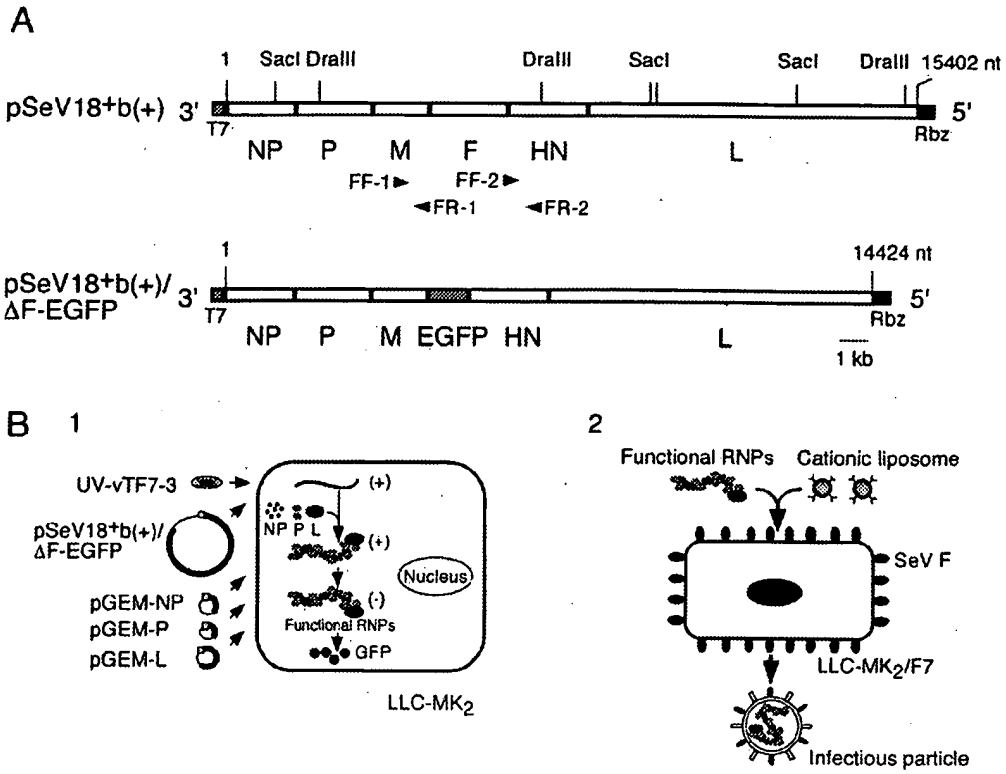

FIG. 1. System for generating the F-defective SeV vector from a cloned SeV cDNA. (A) Schematic representation of the organization of the plasmids pSeV18$^+$b(+), carrying full-length SeV cDNA, and pSeV18$^+$b(+)/ΔF-EGFP, carrying an F-defective SeV cDNA with an EGFP reporter gene. The restriction sites used for construction of pSeV18$^+$b(+)/ΔF-EGFP are indicated. Primers used for PCR amplification are indicated by arrows. T7, T7 promoter; Rbz, hepatitis deltavirus ribozyme sequence; nt, nucleotides. (B) Schematic representation of the two-step procedure for recovery of the F-defective SeV vector. (Panel 1) In the first step, the functional RNPs are recovered in LLC-MK$_2$ cells by using the four plasmids driven by a recombinant vaccinia virus expressing T7 RNA polymerase which had been inactivated with psoralen and long-wave UV light (UV-vTF7-3). (Panel 2) In the second step, RNPs are introduced via a cationic liposome to F-expressing LLC-MK$_2$ cells (LLC-MK$_2$/F7) and produce infectious F-defective virions.

Fig. 1A). The two amplified fragments were digested with *Bsm*I-*Eco*T22I and *Eco*22TI-*Bgl*II, respectively, and ligated with the *Bsm*I-*Bgl*II fragment of pUC18/Sac to generate pUC18/SacΔF. The EGFP gene was amplified by PCR from pEGFP-N1 (Clontech, Palo Alto, Calif.) using a pair of *Nsi*I- or *Ngo*MIV-tagged primers (5′-ATGCATATGGAGATGCGGTTTTGGCAGTAC-3′ [sense] and 5′-TGCCGGCTAATTATTACTTGTACAGCTCGTC-3′ [antisense]). The amplified fragment of EGFP was digested with *Nsi*I and *Ngo*MIV and cloned into the *Nsi*I-*Ngo*MIV sites of pUC18/SacΔF to generate pUC18/SacΔF-EGFP. The 3.4-kb *Dra*III fragment of pUC18/SacΔF-EGFP was replaced with the 4.4-kb *Dra*III fragment of pSeV18$^+$b(+) to generate pSeV18$^+$b(+)/ΔF-EGFP. For the plasmid expressing F protein by the Cre/*loxP*-inducible expression system (1), the 1.8-kb *Sty*I-*Bst*UI fragment of pSeV18$^+$b(+) containing the F gene was blunt ended and inserted into the *Swa*I site of pCALNdLw (1) to generate pCALNdLw/F.

Establishment of F-expressing LLC-MK$_2$/F7 cells. LLC-MK$_2$ cells were transfected with pCALNdLw/F using the mammalian transfection kit (Stratagene) as specified by the manufacturer. G418 (400 μg/ml)-resistant clones were selected after 3 weeks. Expression of F protein was confirmed by infecting the clones with AxCANCre at a multiplicity of infection (MOI) of 3 and analyzed by Western blotting with anti-F monoclonal antibody (MAb) f236 (30) after 3 days. F protein expression on the cell surface was analyzed by flow cytometry after immunostaining with anti-F MAb and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G.

Recovery and amplification of the F-defective SeV vector. Approximately 10^7 LLC-MK$_2$ cells seeded in a 10-cm-diameter dish were infected with psoralen- and long-wave UV-treated vTF7-3 at an MOI of 2. After a 1-h incubation at room temperature, the cells were washed three times with MEM and transfected at room temperature with a plasmid mixture containing pSeV18$^+$b(+)/ΔF-EGFP (12 μg), pGEM-NP (4 μg), pGEM-P (2 μg), and pGEM-L (4 μg) (7) in 110 μl of Superfect transfection reagent (Qiagen, Tokyo, Japan). The transfected cells were maintained for 3 h in 3 ml of OptiMEM (Gibco-BRL) plus 3% FCS, washed three times with MEM, and incubated for 60 h in MEM containing araC (40 μg/ml). GFP expression by the transfected cells was examined by fluorescence microscopy to validate the formation of RNPs inside of the cells. The transfected cells were collected by centrifugation at 1,000 × *g* for 5 min, resuspended in OptiMEM (10^7 cells/ml), and lysed by three cycles of freezing and thawing. Subsequent RNP transfection was performed by mixing the lysate (10^6 cells/100 μl) with 75 μl of OptiMEM and 25 μl of DOSPER (Boehringer Mannheim, Germany) for 15 min at room temperature and then transfecting it into F-expressing LLC-MK$_2$/F7 cells in a 24-well plate. At 24 h after the transfection, the cells were washed three times with MEM and incubated for 3 to 6 days in MEM containing araC (40 μg/ml) and trypsin (7.5 μg/ml). The spread of GFP-expressing cells to neighboring cells was examined by fluorescence microscopy. Virus yield is expressed in PFU and cell infectious units (CIU) (15).

Analysis of viral genomic RNA. Total viral RNA from the F-defective SeV vector or wild-type SeV was isolated using a QIAamp viral RNA mini kit (Qiagen), separated on a 2.2 M formaldehyde–1% agarose gel, transferred to a Hybond N$^+$ membrane (Amersham Pharmacia Biotech, Tokyo, Japan), and hybridized with an F or HN DNA probe generated with a DIG DNA labeling and detection kit (Boehringer). The probes for the F or HN gene were prepared from a 1.8-kb *Sty*I-*Bst*UI or a 1.8-kb *Hha*I-*Dra*I fragment of SeV18$^+$b(+), respectively.

Immunoelectron microscopy. Virus obtained by ultracentrifugation at 10,000 × *g* for 30 min was resuspended in phosphate-buffered saline (PBS) as 10^9 PFU/ml, dropped onto microgrids, dried at room temperature, and fixed with 3.7% formaldehyde for 15 min. Then the grids were treated with anti-F or anti-HN (HN-2) (20) MAb for 60 min, washed three times with PBS, and reacted with gold colloid-labeled anti-mouse immunoglobulin G for 60 min. Treated grids were then washed with PBS, dried, and stained with 4% uranium acetate for 2 min for electron microscopic examination with a JEM-1200EXII instrument (Nippon Denshi, Tokyo, Japan).

Gene transfer to primary cultures of rat cerebral cortex cells. Primary cultures of rat cortical neurons were prepared from E18.5 embryos as described previously (2, 11). Dissociated cells were plated at a density of 80,000 or 100,000/well in eight-well culture slides coated with poly-D-lysine (Becton Dickinson Labware, Bedford, Mass.). The cells were cultured at 37°C in a 5% CO_2 atmosphere for 5 days in neural basal medium enriched with B27 supplement (Gibco-BRL). The F-defective SeV vector was infected at an MOI of 5 and incubated for 3 days. To identify neuronal cells, cells were fixed with 2% paraformaldehyde at room

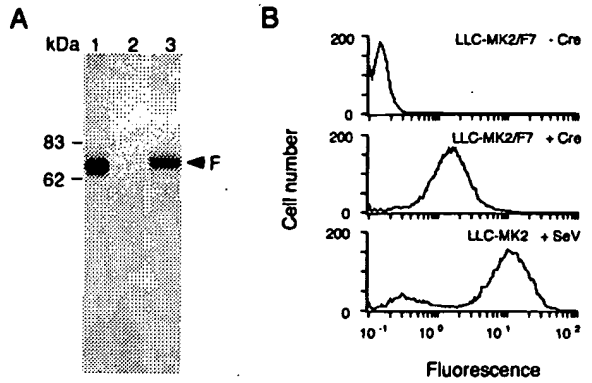

FIG. 2. Inducible expression of F protein in LLC-MK_2/F7 packaging cells. (A) Western blot analysis using anti-SeV F (f-236) MAb. Lanes: 1, LLC-MK_2 infected with wild-type SeV (MOI = 1) for 24 h; 2, LLC-MK_2/F7; 3, LLC-MK_2/F7 infected with adenovirus AxCANCre (MOI = 3) and incubated for 3 days. (B) Flow cytometry analysis of cell surface proteins. Expression of F protein on the packaging cells was examined with the anti-SeV F (f-236) MAb. LLC-MK_2/F7 without induction (top panel), LLC-MK_2/F7 infected with AxCANCre (middle panel), and LLC-MK_2 infected with wild-type SeV (bottom panel) are shown.

temperature for 15 min and immunostained with anti-MAP2 MAb (Boehringer-Mannheim). Immunocytochemistry was performed by indirect-immunofluorescence microscopy (10) with a confocal microscope system (MRC 1024; Nippon Bio-Rad, Tokyo, Japan) using a 470- to 500-nm and 510- to 550-nm excitation band-pass filter on an inverted microscope (Diaphot 30; Nikon, Tokyo, Japan).

Vector injection into rat brain. Female rats, F334/DuCrj (6 weeks old) (Charles River, Ontario, Canada) were anesthetized by intraperitoneal injection of Nembutal (5 mg/kg) and secured on a stereotaxic frame (model 900; David Koph Instruments, Tujunga, Calif.). For intraventricular injection, the burr hole was opened at 5.2 mm off the interaural line toward the bregma and 2.0 mm off lambda toward the right ear. The needle (30 gauge) was inserted 3.6 mm below the surface of the dura. A 20-μl volume of vector suspension (2×10^7 CIU) was injected into the lateral ventricle or hippocampus region.

RESULTS

Construction of F-defective SeV cDNA. F-defective SeV cDNA was constructed by replacing the F gene with an EGFP reporter gene (Fig. 1A). GFP expression was detectable in a single living cell, which allowed us to confirm the successful recovery of RNPs of F-defective SeV inside of such cells.

Construction of a packaging cell line that expresses SeV F protein. SeV F protein is required for the formation of infectious SeV particles. Therefore, recovery of SeV from the RNA genome lacking F gene must be complemented with this gene in *trans*. We therefore constructed an F-expressing packaging LLC-MK_2 cell line with a Cre/*loxP*-inducible expression system. LLC-MK_2 cells were transfected with plasmid pCALNdLw/F, where the F gene is located under the stuffer *neo* sequence flanked by a pair of *loxP* sequences, and stable Neo^r clones were isolated. To these Neo^r clones, a recombinant adenovirus vector, AxCANCre (14), that expresses Cre recombinase was added. Of 15 clones, 7 expressed F protein inducibly; the clone that showed the highest F protein expression (Fig. 2A) was designated LLC-MK_2/F7 and used as a packaging cell line for the F-defective SeV vector. Flow cytometry analysis showed the presence of F protein on the surface of LLC-MK_2/F7 cells (Fig. 2B). The amount of this protein was approximately one-seventh of that on LLC-MK_2 cells infected with wild-type SeV under the same experimental conditions.

Recovery of functional RNPs from an F-defective cDNA. Conventionally, recombinant SeV with the wild-type genome were recovered from cloned cDNAs after infectious particles were rescued in cultured cells and further amplified in embryonated hen eggs or in cultured cells (15). Since infectious particles were not generated from cDNA lacking the F gene in non-F-expressing cells, we have devised a novel rescue procedure which consists of two steps (Fig. 1B). The first step was to recover RNPs of the F-defective RNA genome in LLC-MK_2 cells by using an F-defective cDNA clone and the three plasmids expressing NP, P, and L proteins. GFP-expressing cells were the only RNP-expressing cells on the plate, because such cells were observed only when these four materials were co-transfected into LLC-MK_2 cells. The second step was to transfect RNP into the F-expressing packaging cell line and to collect infectious particles from the supernatants. To raise the efficiency of recovery of RNPs in the first step, we adapted a vaccinia virus vTF7-3 (9) treated with psoralen and long-wave UV irradiation. This treatment inactivated the replication capability of the viruses without impairing their infectivity and T7 RNA polymerase expression. We estimated the recovery frequency by using wild-type SeV cDNA and inoculating the diluted lysates of transfected cells into embryonic hen eggs. With a previous recovery procedure, 1 CIU was detected from 10^5 transfected cells (15). However, with the improved protocol, 1 CIU was detected from only 10^3 cells, indicating an improvement of nearly 100-fold. As for the F-defective SeV cDNA, the numbers of GFP-expressing cells were scored to estimate the efficiency of recovery of functional RNP. Under these conditions, these cells were detected in approximately 1 in 10^5 transfected cells.

FIG. 3. Specific production of the F-defective SeV vector in F-expressing packaging cells in a trypsin-dependent manner. LLC-MK_2 cells (A) or AxCAN Cre-infected LLC-MK_2/F7 cells (B and C) were infected with the F-defective SeV vector and incubated in the presence (A and C) or absence (B) of trypsin. GFP expression by the infected cells was observed by fluorescence microscopy 3 days after infection.

The F-defective SeV vector is specifically propagated in a packaging cell line in a trypsin-dependent manner. The lysates containing functional RNPs were obtained by freeze-thaw cycles, mixed with cationic liposome, and transfected into LLC-MK_2/F7 or LLC-MK_2 cells. The transfected cells were cultured in the presence or absence of trypsin. The infectious virus particles were recovered only from LLC-MK_2/F7 cells cultured with trypsin, suggesting the rescue of infectious virus particles in these cells. The efficiency of recovery at this point was at least 1 CIU from 10^5 transfected cells. In LLC-MK_2/F7 cells cultured in the absence of trypsin or in LLC-MK_2 cells, GFP-expressing cells were detected but did not spread to neighboring cells (Fig. 3). These results showed that the propagation of the F-defective SeV vector and the formation of infectious virus particles are specific to the F-expressing packaging cells and are dependent on trypsin-cleavage. The infectious titer of particles recovered from supernatants of the packaging cells ranged from 0.5×10^8 to 1.0×10^8 CIU/ml.

Confirmation of the genome structure and ultrastructure of the F-defective SeV vector. To examine the genome structure, total RNA from the F-defective SeV vector or wild-type SeV was prepared and analyzed by Northern blot analysis. Probing with the HN gene detected a clear genomic RNA in both F-defective SeV vector and wild-type SeV, but the F-defective SeV vector was smaller than the wild type. When the F gene was used as a probe, no signal was obtained from the F-

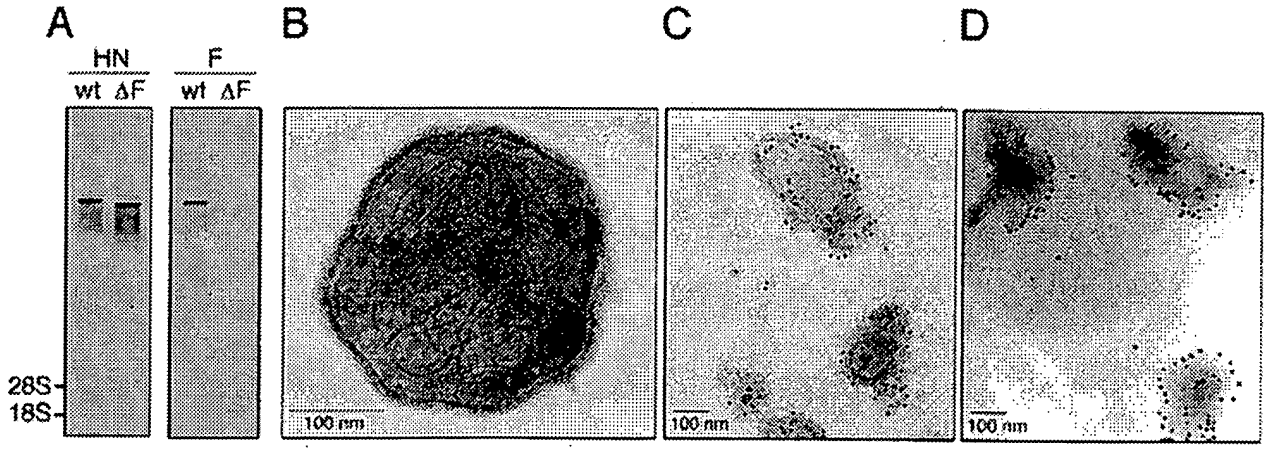

FIG. 4. Structural characterization of the F-defective SeV vector. (A) Northern blot analysis of the RNA genome structure. RNAs from wild-type SeV (wt) and the F-defective SeV vector (ΔF) were prepared and hybridized with cDNA probes of HN (left panel) or F (right panel). The positions of 28S and 18S rRNA are shown. (B to D) Electron microscopic ultrastructure of viral particles. The F-defective SeV vector was negatively stained with phosphotungstic acid (B). The ultrastructure of virus particles after labeling with anti-F (C) or anti-HN (D) MAb and gold-conjugated goat anti-mouse immunoglobulin G is shown.

defective SeV vector but a clear signal was obtained from wild-type SeV (Fig. 4A). The reverse transcription-PCR analysis confirmed the existence of the EGFP gene in the F-deleted region of the F-defective SeV vector (data not shown). These results confirmed that the F-defective SeV vector contains an RNA genome lacking the F gene. Electron microscopic examination of the F-defective SeV vector revealed internally located helical RNP-like structure and an envelope studded with spike-like structures (Fig. 4B). Immunoelectron microscopic examination located the F and HN proteins on the surface of the F-defective SeV vector (Fig. 4C and D).

The F-defective SeV vector efficiently delivers and expresses the EGFP gene in variety of cell types. When primary cultures of neuronal cells derived from fetal rat cerebral cortex were infected with the F-defective SeV vector carrying the EGFP reporter gene at an MOI of 5, nearly 100% of the microtubule-associated protein 2 (MAP2)-positive cells expressed the EGFP reporter gene (Fig. 5A to C). Also, the vector infected and strongly expressed the EGFP gene in almost 100% of normal human hepatocytes, lung microvascular endothelial cells, and smooth muscle cells at an MOI of 3 (Fig. 5D to I). EGFP fluorescence of the infected cells was seen at least from 10 h to 10 days after vector infection. Furthermore, GFP expression was observed in nondividing neuronal cells or ependymal cells of the lateral ventricle when the vector was stereotaxically injected into the hippocampal region or an intraventricular region of rat brain, respectively (Fig. 6). Gene introduction into ependymal cells is of value, since it was reported recently that these cells could be neural stem cells that generate migratory neuronal precursor cells (13). These results showed that the F-defective SeV vector is capable of efficient infection and strong expression of foreign genes in a wide spectrum of cells and tissues.

DISCUSSION

The development of a reverse genetic system has enabled the genetic engineering of negative-strand RNA viruses. This

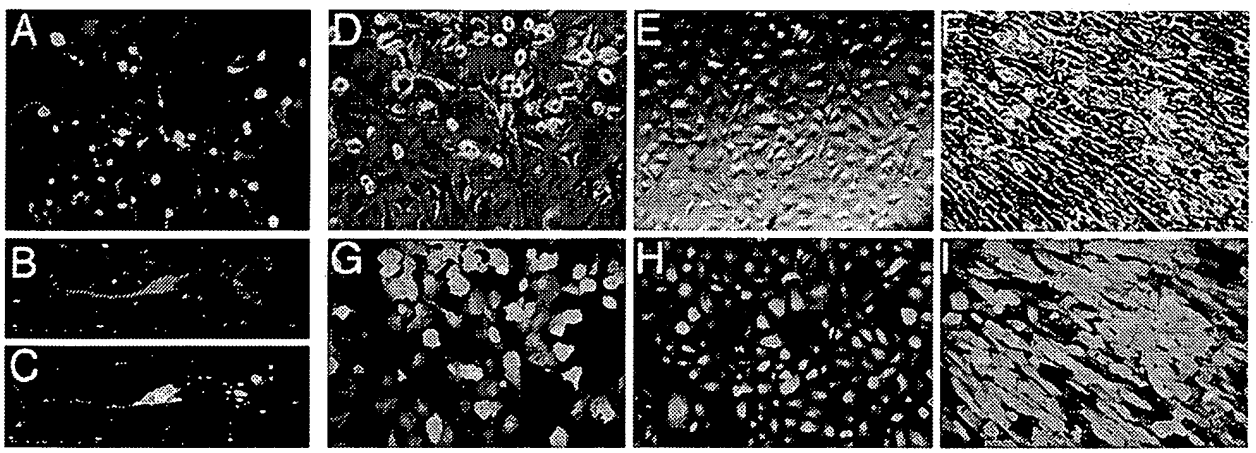

FIG. 5. Introduction and expression of the EGFP gene by the F-defective SeV vector in a variety of cell types in vitro. (A to C) GFP expression by primary neuronal cells derived from rat cerebral cortex 5 days after infection with the vector at an MOI of 5 at lower (A) and higher (C) magnification and immunostained with anti-MAP2 antibody (B). (D to I) Normal human hepatocytes (D and G), normal human lung microvascular endothelial cells (E and H), and normal human smooth muscle cells (F and I) were infected with the F-defective SeV vector at an MOI of 3. GFP expression was observed 3 days after infection (G to I).

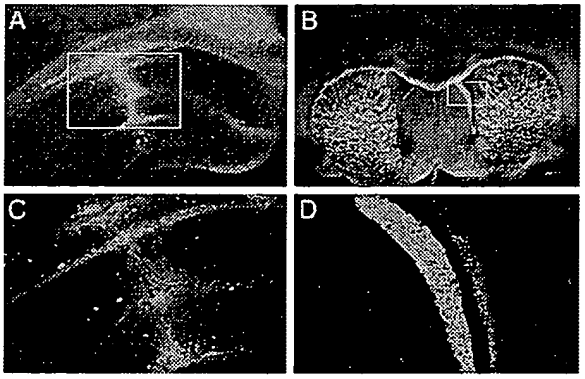

FIG. 6. Gene introduction into the rat central nervous system. The F-defective SeV vector carrying the EGFP gene was injected into rat brain. GFP expression was observed 4 days after vector injection. Fluorescent photomicrographs at lower (A and B) and higher (C and D) magnifications of pyramidal cells of the CA1 region in the hippocampus and ependymal cells of the lateral ventricle.

system has been used to analyze the function of viral genes and to construct recombinant viruses which express foreign proteins. In this study, we made an improvement to this system by devising a new method to generate the F-defective SeV vector from a cloned cDNA of a defective RNA genome. This is the first report on constructing a replicon-based RNA vector in the family *Paramyxoviridae* which replicates in infected cells but does not infect neighboring cells. The improvements achieved in this study are (i) optimization of RNP recovery efficiency by using a UV-inactivated recombinant vaccinia virus expressing T7 RNA polymerase, (ii) construction of an inducible F-expressing packaging LLC-MK_2 cell line supplemented with the F protein in *trans*, and (iii) development of a transfection process for RNP recovered from LLC-MK_2 cells. An attempt to recover the F-defective SeV vector directly in the F-expressing packaging cell line by transfecting F-defective cDNA together with three plasmids expressing NP, P, and L proteins was unsuccessful. Our observation on the gross reduction in F protein expression after vaccinia virus infection of packaging cells suggests that this protein was depleted during this approach (data not shown). The fact that the F-defective SeV vector cannot spread to F-nonexpressing cells indicates that F protein is indispensable for viral infection. Since this system requires the NP, P, and L genes for self-replication and transcription of RNP, a variety of similar self-replicating SeV vectors defective in M, HN, and/or a combination of M, HN, and F genes could be designed if proper complementing cell lines are constructed. Further, we speculate that the strategy developed in this study for rescuing defective viruses is applicable to other negative-strand RNA viruses and represents an innovative method for generation of novel types of vectors.

As to paramyxoviruses carrying defective genome, measles virus defective in M gene were isolated from the brains of subacute sclerosing panencephalitis patients and generated by reverse genetic techniques (4). These viruses were not able to generate progeny viral particles because of the defect in viral envelope assembly but did spread by cell-to-cell fusion. Defective interfering particles of negative-strand RNA viruses which are defective in several viral genes and interfere with the replication of nondefective virus are generated in nature (35). Furthermore, minigenomes in which the entire coding region was replaced with a reporter gene were constructed by genetic engineering in negative-strand RNA viruses (5, 25, 31). Defective interfering particles and minigenomes require helper virus for their replication and virion assembly. The F-defective SeV vector reported in this study is independent of helper virus for its reproduction and is able to self-replicate in infected cells. In the family *Rhabdoviridae*, generation of G-gene-deficient viruses which carry human immunodeficiency virus (HIV) receptor and coreceptor genes has been performed in the vesicular stomatitis virus and rabies virus groups (19, 29). These pseudotyped rhabdoviruses were constructed specifically for targeting to cells infected with HIV-1. Vesicular stomatitis virus has also been used as a vaccine vector (27).

The F-defective SeV vector has several advantages over existing vectors as a gene delivery system for human treatments. (i) SeV is a murine parainfluenza virus, and pathogenicity to humans has not been reported. (ii) This vector replicates exclusively in the cytoplasm of infected cells and does not go through a DNA phase; therefore, there is no concern about unwanted integration of foreign sequences into chromosomal DNA. (iii) This vector has shown a high efficiency of gene transfer and expression of a foreign reporter gene to a wide spectrum of cells and tissues, which is comparable to SeV vectors derived from the wild-type genome. The highest level of expression in mammalian cells has been found in a recombinant SeV expressing HIV-1 envelope glycoprotein gp120 (36). For expression of foreign genes in recombinant F-defective SeV vectors, the genes can be designed as the 3′ proximal first gene of the viruses. A vector with a 3.2-kb foreign gene has been successfully recovered (data not shown). (iv) This vector is not likely to generate wild-type virus in a packaging cell line, since homologous recombination between RNA genomes has not been observed in nonsegmented negative-strand RNA viruses (33). The following studies have confirmed this idea. The F-defective SeV vector was inoculated into embryonated hen eggs or into non-F-expressing LLC-MK_2 cells. The allantoic fluids or the culture supernatants were harvested several days after the vector infection and reinoculated into LLC-MK_2 cells. The presence of infectious viruses in infected cells was examined by GFP expression or immunostaining with an anti-SeV serum. Repeated studies have detected no infectious particles.

Replicon-based vectors derived from positive-strand RNA viruses such as *Sindbis virus* and *Semliki Forest virus* expressed foreign genes with high efficiency, but foreign genes were rapidly lost on passaging of infected supernatant. Also, these vectors had severe cytopathic effects on infected cells (8, 17). The F-defective SeV vector developed in this study is likely to overcome these disadvantages of positive-strand RNA vectors.

One application of this vector is for human gene therapy. The high-level expression of therapeutic genes in wide varieties of cell types, including nondividing types, and the potential safety to humans suggest that this novel vector has great potential for use in transient gene therapy at least (6). Another potential application is in the development of vaccines. This vector resembles DNA vaccines because of its ability to express epitopes of foreign proteins without generating infectious viruses. Therefore, this vector is useful for the design of improved attenuated vaccines. The applications to the treatment of human diseases are now in progress.

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AB Sendai virus (SeV) is an enveloped virus with a negative sense **genome** RNA of about 15.3 kb. We previously established a system to recover an infectious virus entirely from SeV cDNA and illustrated the feasibility of using SeV as a novel expression **vector**. Here, we have attempted to insert a series of foreign genes into SeV of different lengths to learn how far SeV can accommodate extra genes and how the length of inserted genes affects viral replication in cells cultured in vitro and in the natural host, mice. We show that a gene up to 3.2 kb can be inserted and efficiently expressed and that the replication speed as well as the final virus titers in cell culture are proportionally reduced as the inserted gene length increases. In vivo, such a size-dependent effect was not very clear but a remarkably attenuated replication and pathogenicity were generally seen. Our data further confirmed reinforcement of foreign gene expression in vitro from the V(-) version of SeV in which the accessory V gene had been knocked out. Based on these results, we discuss the utility of SeV **vector** in terms of both efficiency and safety.

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